Evaluation of Salivary IgA as a Potential Stress Biomarker for Use in Military Training

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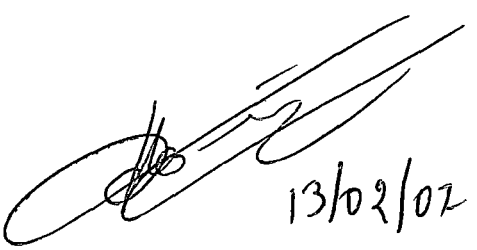

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ABSTRACT

Exercise and psychological stress can cause a transient decrease in salivary IgA (s-IgA) and regular intense physical activity can result in a chronic decrease in s-IgA. Epidemiological evidence supports the concept of an association between low levels of s-IgA and the incidence of upper respiratory tract infections (URTI) and overtraining syndrome (OTS). However, the relationships are not strong.

The impact of stress on immune function is of interest to the Australian Defence Force. Soldiers are often subjected to high levels of physiological and psychological stress, and thus susceptible to impaired mucosal immune function and an increased risk of URTI, which could reduce their performance. Newly enlisted recruits may also be susceptible to OTS as they do not necessarily have a very high level of physical fitness. The purpose of this study was to assess the usefulness of s-IgA as an indicator of mucosal immune function, the effect of a variety of stressors on mucosal immune function and its relationship to the incidence of symptoms of illness and OTS.

Preliminary work raised some concerns regarding the reliability of s-IgA. The main weaknesses identified were the possible effects of diurnal variation, the large biological variation, the standardisation of the s-IgA assay, and the collection, handling and storage methods of saliva samples. These issues were addressed by several preliminary experiments and recommendations were made (Chapter 2). Although s-IgA can be expressed in several ways (as a concentration, a secretion rate, a ratio to secreted protein, or as a ratio to osmolality), only s-IgA secretion rate and s-IgA:osmolality ratio are used in the experimental chapters 3 to 5.

An 82 km ultra-endurance running race was the first opportunity to test the methods developed for the collection, storage and transport of saliva under "field conditions" (Chapter 3). The race resulted in a transient decrease in s-IgA and several other immune parameters.

Soldiers who engaged in two hours of load carriage (Chapter 4) experienced changes in heart rate, metabolic rate, core temperature and perceptions of effort and thermal strain. These changes were greater with higher environmental temperatures and humidity. Only the hottest, most humid environment resulted in a significant decline of s-IgA. Therefore, it seems that if exercise at an intensity that by itself does not cause a significant mucosal immunosuppression is performed in a harsh climate, the resulting hyperthermia can magnify the s-IgA response.

A six week army common recruit training course (Chapter 5) resulted in a high incidence of mucosal immunosuppression and self-reported symptoms of URTI. There was also a significant incidence of OTS symptoms.

Despite these findings, this work does not lend strong support to the notion that s-IgA could be a practical tool to monitor mucosal immune function or physiological strain in the military. Nor does it seem a good predictor of ill health or early states of OTS.

List of abbreviations

ADF	Australian Defence Force
ADHREC	Australian Defence Human Research Ethics Committee
ACRT	Army common recruit training
ANS	Autonomic nervous system
ANOVA	Analysis of variance
ARTC	Army recruit training centre
BD	Body density
$BF_{\%}$	Percentage body fat
BM	Body mass
BMI	Body mass index
BMR	Basic metabolic rate
CFA	Combat fitness assessment
CMR	Cradle Mountain run
CRM	Certified reference material
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CV_A	Assay coefficient of variation
CV_I	Within-subject coefficient of variation
CV_G	Between-subject coefficient of variation
CV_T	Total variation
DOMS	Delayed onset muscle soreness
DPCU	Disruptive pattern combat uniform
DSTO	Defence Science and Technology Organisation
ELISA	Enzyme-linked immunosorbent assay
EX DW	Exercise Dusty Warrior
Frt	Ferritin
FTCR	Free testosterone to cortisol ratio
HD	Hot and dry conditions in the climate chamber
HR_{max}	Maximum heart rate
HW	Hot and wet conditions in the climate chamber
IFCC	International Federation of Clinical Chemists
IgA	Immunoglobulin A

II	Index of individuality
IL	Interleukin
IQC	Internal quality control
NSAID	Non-steroid anti-inflammatory drug
OTS	Over training syndrome
PAL	Physical activity level
PENIA	Particle enhanced nephelometric immuno-assay
POMS	Profile of mood states
QC	Quality control
RH	Relative humidity
RPE	Rate of perceived exertion
SD	Standard deviation
SEM	Standard error of the mean
SC	Secretory component
SIgA[1]	Secretory IgA
s-IgA	Salivary immunoglobulin A (S-IgA if used at the start of a sentence)
T	Temperate conditions in the climate chamber
T_{core}	Core body temperature
TNF_{α}	Tissue necrosis factor alpha
TEE	Total energy expenditure
URTI	Upper respiratory tract infection
VO_{2max}	Maximum oxygen uptake

[1] The use of the abbreviation "s-IgA" for salivary IgA should not be confused with the abbreviation "SIgA" for secretory IgA. At the start of a sentence "S-IgA" is used for salivary IgA.

GENERAL INTRODUCTION

1.1 The immune system

The body is under constant attack by pathogenic organisms such as bacteria and viruses. Invasion by these organisms can result in infection and disease. To protect itself, the body has a built-in defence: the immune system. Its essential function is illustrated by the often-lethal results of an impaired immune system, for instance due to genetic defects or infection by the AIDS virus (Elliott and Elliott, 1997). The immune system consists of two major components, the non-specific or innate immune system, and the specific or adaptive immune system.

The innate immune system is the body's first line of defence against pathogens. It is present from birth until death; it is always prepared and responds almost immediately; it has no memory because it does not remember if a pathogen has been encountered before. It consists of three components - physico-chemical, humoral, and cellular. Physical barriers that prevent the entry of micro-organisms are the external body membranes (intact skin and mucous membranes), secretions, and cilia. Mucous membranes line all body cavities that are in contact with the exterior - the digestive, respiratory, urinary, and reproductive tracts. Secretions continuously wash and cleanse mucosal surfaces and cilia help with the removal of debris and foreign matter. Although these surface barriers are very effective, they are breached occasionally. When micro-organisms penetrate them, the humoral and cellular parts of the innate immune system are called upon. These internal, non-specific mechanisms use cellular and chemical devices to protect the body. Anti-microbial proteins (e.g. complement, opsonins, and interferon), proteolytic enzymes, as well as phagocytes (e.g. neutrophils) and natural killer cells inhibit the spread of pathogens throughout the body.

The specific immune system is not present at birth but is gained during maturation and by exposure to pathogens. It recognises specific pathogens and acts to immobilise, neutralise, or destroy them. Unlike the non-specific system, the specific immune system must be "primed" by an initial exposure to an antigen before it can protect the body against further attacks by the same antigen. However, once it has learned to identify an antigen, future defences can be mobilised more quickly. An antigen is any foreign substance that generates a response by the immune system. The specific immune response is characterised by three aspects. It is antigen-specific as it recognises and is directed against particular antigens. It is not restricted to the initial infection site,

therefore, it is systemic. It has memory because after an initial exposure it recognises and mounts even stronger attacks on a previously encountered antigen. The specific immune system has two separate but overlapping components: (1) the cellular or cell-mediated immune system, and (2) the humoral or antibody-mediated immune system. Although these systems respond to virtually the same antigens, they do it in different ways.

The response provided by the cellular immune system uses living cells as protective elements - lymphocytes. They target virus or parasite infected tissue cells, cancer cells and cells of foreign grafts. The lymphocytes act either directly by lysing the foreign cells, or indirectly by releasing chemicals that enhance an inflammatory response or activate other lymphocytes or macrophages to lyse the foreign cells.

Immunoglobulins are an important component of humoral immunity to infectious agents such as bacteria, bacterial toxins, viruses and parasites. Immunoglobulins circulate freely in body fluids (humours) like serum, lymph, tissue fluids and in mucosal secretions such as saliva. An antibody is an immunoglobulin produced in response to an antigen that has the ability to bind specifically to that particular antigen. Immunoglobulins are produced by activated B-lymphocytes (B-cells) and their descendants, the antibody-forming cells and plasma cells found in blood, lymphoid organs and other tissues. Because each lymphocyte is only capable of recognising one of an infinite number of antigens, an adequate immune response depends on the proliferation of antigen-specific cells.

Antibodies themselves do not destroy pathogens. Their primary role is to bind to antigens, initiating a variety of responses that act, both directly and indirectly, to combat the foreign agent carrying the antigen. Direct antibody actions include binding to antigens on micro-organisms and inhibiting their access to the host's cells. More prevalent, however, are indirect actions that stimulate the recruitment, direction, recognition and binding of antibacterial effector mechanisms such as complement, phagocytes, or other cytotoxic cells which themselves cannot recognise foreign organisms. This process results in lysing of bacterial and virally infected cells. Other functions of antibodies include a direct binding to bacterial toxins, thus neutralising their deleterious effect, interfering with movement and binding of bacteria to the host's cells, and inhibiting the uptake of essential nutrients by bacteria. Antibodies bind directly to extra-cellular viruses, preventing them from gaining entry to the body and facilitate recognition and killing by cytotoxic lymphocytes. Antibodies also enhance phagocytosis and cytotoxicity against parasites.

Antibodies are grouped into five classes, each with slightly different structure and function. These five classes are, in order of abundance in the body, IgG, IgA, IgM, IgD, and IgE. The antibodies of each class have slightly different biological roles and locations in the body. IgA, the second most abundant immunoglobulin, exists either as a monomer, a single IgA molecule, or as a dimer, in which two IgA molecules are joined together by a polypeptide (J chain). IgA is abundant in serum and is the dominant antibody in the mucosal immune system. In humans IgA occurs as two subclasses, IgA1 that predominates in serum and IgA2 that predominates in most mucosal secretions.

The secretory or mucosal immune system is made up of a network of immune structures that form the first line of defence at mucosal surfaces. It incorporates the gut-associated lymphoid tissue, uro-genital tract mucosa, lacrimal glands, lactating mammary glands and in the respiratory tract, the bronchus-associated lymphoid tissue, nasal-associated lymphoid tissue and salivary glands. When antigens present at a mucosal surface specific antibodies are secreted. The main mucosal antibody found in secretions is secretory IgA (SIgA). It is a dimer, containing part of a polypeptide called secretory component (SC). Secretory IgA is found in saliva, sweat, lung fluids, bronchial fluid, gastrointestinal secretions, tears, breast milk, and vaginal secretions. As most infectious agents enter the host via the mucosal membranes, a secretory IgA deficiency represents a reduced level of protection for the body, and an increased risk of infection. Therefore, a decreased concentration of secretory IgA is a potential indicator of increased disease risk.

Secretory IgA protects the mucosal surfaces by several mechanisms: exclusion (Lamm, 1976), neutralisation (Mazanec et al., 1992), and elimination (Kaetzel et al., 1991). Exclusion is the mechanism whereby secretory IgA antibodies bind with exogenous substances at the luminal surface and prevent their attachment to the epithelial cells of the mucosa or entry into the body across the epithelium (Lamm, 1976).

The protective function of secretory IgA is not limited to the external secretions. Because secretory IgA migrate from plasma cells across epithelial cells to the lumen, they have the opportunity to encounter pathogens infecting the same cell. Specific secretory IgA antibodies can neutralise viruses at intracellular level by binding to viral proteins within the mucosal epithelium, thereby limiting or disrupting viral replication (Mazanec et al., 1993b). Thus, secretory IgA can not only prevent infection by keeping viruses from invading cells, it also appears capable of aiding recovery after infection.

Another site of secretory IgA defence is the lamina propria. Because secretory IgA is produced by most of the plasma cells in the mucosal lamina propria, a high local concentration of antibodies is present in the extra-cellular fluid. These antibodies have an opportunity to combine, at the basolateral surface of the mucosal epithelium, with specific antigens that have crossed it, or have been produced in the sub-mucosa and would be present in the same extra-cellular fluid (Lamm et al., 1995). Rather than being absorbed into the circulation, these immune complexes can be transported across the epithelium into the mucosal lumen for elimination and excretion (Mazanec et al., 1993b).

1.2 Salivary IgA

The salivary glands are the main source of salivary IgA (s-IgA)[2] (Miletic et al., 1996). Another source of secretory IgA in the upper respiratory tracts is the tonsils. The release of s-IgA into saliva is a well-regulated process (Mayhall, 1975). It is produced by IgA-secreting plasma cells that migrate, for instance from the gut and the uro-genital tract, to the salivary glands. There they reside beneath the epithelial cells which make up the surface of the mucous membranes (Brandtzaeg, 1989). Since these cells adhere to their neighbours by tight junctions, diffusion of macromolecules like secretory IgA (SIgA) across the epithelial layer is prevented and thus requires transport (Mazanec et al., 1993a). The molecule which acts as both a receptor and trans-epithelial transporter for IgA is secretory component (SC). When secreted from the plasma cell, the J-chain of the dimeric SIgA binds to SC. Next, the SC-SIgA complex is incorporated by endocytosis into the cytoplasm of the epithelial cell, crosses it towards the luminal surface and is finally secreted by exocytosis into the lumen (Brandtzaeg and Prydz, 1984). At this point, the SC-SIgA complex is cleaved, releasing the dimeric IgA with a portion of the SC still attached. This process is shown in Figure 1.

The immune system in general is influenced by a variety of factors and stressors that can be broadly categorised as physical (e.g. exercise, age, gender), environmental (e.g. heat and humidity), psychological (e.g. mood states) and lifestyle factors (e.g. nutrition). Most of these factors can also influence s-IgA secretion (Bratthall and Widerström, 1985; Gleeson, 2000a; Miletic et al., 1996; Pyne et al., 2000). After a discussion on several methodological issues, the following review is limited to the effects of exercise, heat and humidity, and psychological stress.

[2] The use of the abbreviation "s-IgA" for salivary IgA should not be confused with the abbreviation "SIgA" for secretory IgA. At the start of a sentence "S-IgA" is used for salivary IgA.

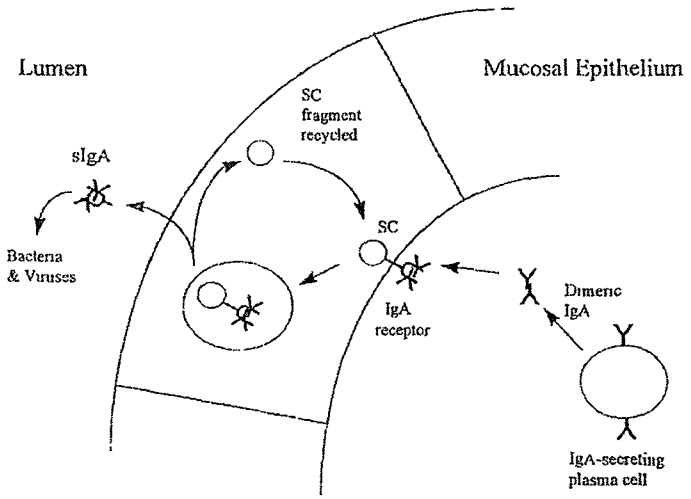


Figure 1: *General scheme of secretory IgA transport across the mucosal epithelium. From Mackinnon (1999a), Advances in Exercise Immunology, page 179. © 1999 by Laurel T. MacKinnon. Reprinted with permission from Human Kinetics (Champaign, IL).*

1.3 Methodological issues

Saliva is the mucosal secretion of choice for the majority of studies of the mucosal immune system. It is more acceptable to donors than blood, urine, tears or lymph because its collection is easy, non-invasive and mostly painless.

Unfortunately, it is often difficult to compare studies on s-IgA because of different collection and storage methods, analytical methods and ways of expressing s-IgA levels. It is important to report the physiological conditions under which saliva is collected because they can have a significant influence on the s-IgA concentration, irrespective of the acute or chronic influence of exercise. Factors to consider are the source of the saliva (i.e. which gland), whether the subject is fasting, and whether saliva flow was stimulated. Careful handling and processing of saliva samples is required during the collection, transport, storage, and analytical phase of experiments because s-IgA could be subject to degradation over time.

1.3.1 Salivary sources

To interpret and compare results on saliva flow rate and s-IgA during exercise, it is important to know if the saliva was whole mixed saliva or saliva from a single gland. The variations in s-IgA levels across studies can sometimes be explained by the fact that

the saliva came from different glands and that the contribution of each salivary gland varies with stimulation (Dawes, 1974). The concentration of IgA is lower in the parotid glands and this may account for lower concentrations of IgA in stimulated saliva secretions where the proportion of parotid secretions is increased to 50% of the total (Gleeson, 2000a). Because whole saliva provides a more complete picture of s-IgA immune status than saliva from a single gland, it is advisable to collect whole saliva rather than single gland saliva (Jemmott and McClelland, 1989).

1.3.2 Saliva flow rate

Saliva flow rate is a major factor governing the rate at which s-IgA is supplied to the oral cavity. Saliva flow rate is under neural control, predominantly by the autonomic nervous system. It is stimulated or influenced by a variety of factors including age, time of day, body position, food ingestion, sensory stimulation (e.g. light vs. dark; smell), drugs, smoking, body position, stress, exercise and degree of hydration (Ben-Aryeh et al., 1984; Dawes, 1974; Ferguson and Botchway, 1980; Gutman and Ben-Aryeh, 1974; Miletic et al., 1996). Parasympathetic stimulation causes vasodilation of the vessels supplying the salivary glands, resulting in an increase in blood flow and higher rates of salivary secretion (Chicharro et al., 1998b). Conversely, stimulation by the sympathetic nervous system, for instance during exercise, causes vasoconstriction of the blood vessels to the salivary glands, leading to a reduction in saliva secretion (Chicharro et al., 1998b). The effects of short, intense exercise on saliva flow rate remain controversial (Chicharro et al., 1998b). Studies at sub-maximal or maximal intensity show no changes in flow rate (Walsh et al., 1999), increased flow rate, or decreased flow rate after exercise (Blannin et al., 1998; Mackinnon and Jenkins, 1993). The few studies examining the effect of prolonged exercise suggest that low-moderate intensity exercise has little effect on saliva flow rate, while prolonged exercise above anaerobic threshold causes a decrease in salivary secretion (Bishop et al., 2000). The fall in saliva flow rate can be explained by a combination of a loss of saliva water by evaporation when breathing through the mouth and a reduction in saliva secretion due to dehydration (Blannin et al., 1998). Assuming saliva flow rate during exercise is affected by hydration levels, studies should indicate whether steps were taken to prevent or to minimise this by regular fluid intake.

Saliva flow rate and s-IgA concentration also depend on whether saliva flow was stimulated and how, and whether the subject was fasting. A commonly used method is the application of citric acid or sour lemon drops to the tongue. Stimulation causes an

increase in saliva flow rate (Dawes, 1974; Miletic et al., 1996), resulting in a decrease in s-IgA concentration (Mandel and Khurana, 1969) and/or an increase in s-IgA secretion rate (s-IgA output can be up to four times higher in stimulated samples as compared to unstimulated samples) (Bratthall and Widerström, 1985). It also causes a variation in the contribution of each salivary gland (Dawes, 1974). In fasting subjects, changes in flow rates may account for the higher levels of s-IgA (Gleeson et al., 1990; Mandel and Khurana, 1969). Therefore, it is essential that studies mention whether stimulated or unstimulated saliva samples were obtained and wether subjects were fasting or not.

1.3.3 The salivary IgA assay method

Changes in assay methods have not had a significant impact on the accuracy of s-IgA measurement (Gleeson, 2000a). Nevertheless, when comparing studies, one has to take into consideration that differences in analytical methods can have an impact on the interpretation of results. Three aspects that need to be considered are the assay method, the source of the calibration material, and the specificity of the antisera. The most commonly used assay is (in-house) enzyme linked immunosorbtion assay (ELISA). Other methods include radial immunodiffusion (Winzer et al., 1999), immunoelectrophoresis (Guhad and Hau, 1996) and nephelometry (Deinzer et al., 2000), which is the method used by our laboratory.

Direct comparison of results from older studies is difficult because of a lack of information on the sources of the calibration material used for the assays (Gleeson, 2000a). In 1994 a certified reference material (CRM 470) was prepared by the International Federation of Clinical Chemists (IFCC). The use of any calibrator standardised against this reference now allows for direct comparison between studies. A literature search revealed, however, that not all recently published reports have referenced CRM 470. The calibration standards used in our laboratory are referenced against CRM 470.

The major analytical difference between reports of s-IgA concentrations is due to the differences in specificity of the antisera (Gleeson, 2000a). S-IgA exists in two forms, IgA1 and IgA2, which are present in almost equal proportion in saliva (Delacroix et al., 1982). Therefore, it is important to select an antiserum that it is able to detect both subclasses. The Dade-Behring antiserum used by our laboratory detects both IgA subclasses (Ralf Evelbauer, Dade-Behring Technical Support Group Plasma Proteins, personal communication, 4 September 2002).

1.3.4 The collection of saliva samples

Differences and errors in the collection method of saliva may explain some of the biological variation and differences between studies. The most common methods are spitting, dribbling (collection of drool), suction, and the use of absorbent swabs (Dawes, 1987). Spitting requires subjects to be seated in a comfortable position, with slightly lowered head, allowing spontaneous saliva flow in the mouth (Miletic et al., 1996). Saliva is expectorated at predetermined time intervals into a receptacle. Dribbling requires subject to be seated with the head tilted forward and the mouth slightly open, allowing saliva to drip from the lower lip into a receptacle (Dawes, 1972). During the last few seconds of the collection time, saliva accumulated in the mouth is spat out. No other conscious movements of the oral musculature are allowed during the collection period. In the suction method, saliva is sucked continuously from the floor of the mouth with a suction tube (or pipette) and allowed to accumulate in a receptacle (Dawes, 1987). Suction tubes are also used to extract saliva directly from specific glands. The method that uses absorbent swabs requires the subjects to hold a small cylinder of a cotton-wool-like substance in the mouth. The saliva absorbed by the swab is removed by centrifugation. Several commercial devices are available, for instance Salivettes (Sarstedt, Numbrecht, Germany), OraSure (Epitope, Beaverton, Oregon), Oracol (Malvern Medical Development Ltd., Worcester, United Kingdom) and Omni-Sal (Saliva Diagnostic Systems Inc., Brooklyn, New York). For the accurate measurement of saliva flow and the calculation of s-IgA secretion rate it is essential that all saliva present in the mouth is collected. This requires the full cooperation of the subject. Imprecise timing of the collection can also introduce errors when measuring the secretion rate of IgA.

It is possible that the s-IgA concentration is influenced by the saliva collection method. Two studies have found s-IgA concentration to be lower in saliva collected by the cotton swab (Salivette) method than in saliva collected by other methods (Aufricht et al., 1992; Strazdins et al., 2005). This was ascribed to an unspecific adsorption of s-IgA to the material of the swab. It is not clear if this problem has been overcome since then by the use of a different material (polyester) for the swab. If not, this method contains a potential source of error. In another study, however, comparing four different collection methods (drooling, suction, OraSure and Oracol) the collection method had only a minor effect on antibody concentration (Nurkka et al., 2003). In any study on s-IgA, the collection method should be accurately described.

A standardised collection method, using Salivettes with polyester swabs, was developed early in this study and used in all related projects. An accurate description of this method can be found in the "methods" section of chapter 2.

1.3.5 The storage of saliva samples

If saliva samples are left at room temperature, s-IgA is possibly subject to proteolysis, the degradation by proteases produced by bacteria in the respiratory tract (Dawes, 1974). Therefore, it is recommended that saliva is either analysed straight away (Butler et al., 1990) or, if this is not possible, frozen immediately, either by snap-freezing in liquid nitrogen or in a -70°C freezer (Nurkka et al., 2003). For added "protection" a protease inhibitor can be added (Ogra et al., 1999). If no freezer is available samples should be placed preferably in dry ice within 30 minutes of collection or otherwise placed directly into normal ice (M. Gleeson, personal communication 01/09/02).

It is possible, especially in field testing situations, that saliva samples are collected in situations where no cold storage facilities are available and ambient temperature is quite high (e.g. remote regions of the tropics). Therefore, to be of any use in those circumstances, it is essential that s-IgA remains stable until samples arrive at the laboratory.

Several studies have investigated how saliva and various salivary proteins are affected by storage conditions (Mortimer and Parry, 1988; Nimmagudda et al., 1997; Stark et al., 1993; Thwe et al., 1999). However, only a few have assessed the stability of s-IgA. In one study, saliva samples were stored at 4°C, -20°C and -70°C, with or without glycerol and a protease inhibitor for various periods (Butler et al., 1990). S-IgA activity to *S. mutans* decreased significantly after one week when stored at 4°C and -20°C. Loss of activity was significant in the first 24 h. The addition of 50% glycerol and storage at -70°C prevented this degradation. Samples stored for 18 months at -20°C lost all antibody activity for *S. mutans*. In another study, s-IgA concentration remained stable for up to 3 months at -30°C, then declined with increasing storage time and after 8 months had decreased by more than 10% in the majority of the samples (Ng et al., 2003). Nurkka et al. (2003) compared the effect of three storage protocols on the concentrations of s-IgA antibodies to pneumococcal capsular antigens. Samples were either snap-frozen with glycerol in liquid nitrogen, stored at +4°C either with or without the addition of protease inhibitors, or stored at -70°C for a week. Samples which were not frozen immediately yielded IgA concentrations approximately 30% lower than the

snap frozen samples. The addition of a protease inhibitor made no difference to this result. However, the advantage of snap-freezing was lost if not applied immediately after collection of the saliva.

From these results it seems that long-term storage can cause some degradation of s-IgA even at –30°C but that storage at –70°C can prevent loss of s-IgA. It is not clear if repeated freeze-thawing cycles affect s-IgA concentration. According to M. Gleeson (personal communication 01/09/02) s-IgA is stable for approximately 5 repeat freeze-thaws providing the sample is not left at room temperature for any more that 10 minutes during each thaw. However, repeated freeze-thaws should possibly be avoided, as the concentration of other proteins (e.g. albumin, IgG) has been shown to decrease by 10% with each additional thaw

1.3.6 Measures of salivary IgA

There remains some controversy and confusion about the best way to express s-IgA levels - as an absolute concentration (in $mg\,L^{-1}$), as a ratio to total protein, as a ratio to albumin, as a ratio to osmolality, or as a secretion rate (in $\mu g\,min^{-1}$). Each of these measures is associated with methodological problems. The lack of uniformity in expressing s-IgA makes comparison of published effects of physical activity on mucosal immunity difficult.

The IgA concentration in saliva is influenced by the saliva flow rate. It is quite common that saliva flow is reduced by exercise-induced dehydration. This, and evaporative loss of saliva water when breathing through the mouth during exercise, can have a concentrating effect on the saliva (Blannin et al., 1998; Stone et al., 1987). If s-IgA output and transport remain constant, absolute s-IgA concentration could be artificially increased because of the reduced saliva volume as a result of the dehydrating effect of the exercise. Conversely, a decreased s-IgA output and transport during physical activity may not be detected. Therefore, reporting the s-IgA concentration can be misleading and make it difficult to interpret the effect of exercise. Absolute s-IgA concentration should only be used when saliva flow rate remains constant.

Expressing s-IgA concentration as a ratio with another salivary solute, which is not actively transported and therefore is insensitive to most physiological changes, should account for changes in saliva water volume. A commonly used indirect quantity for assessing s-IgA levels is the ratio of the s-IgA concentration to the total protein or albumin concentration. Proponents of these measures claim that they give a better indication of the effects of exercise on s-IgA because, compared to s-IgA concentration,

they are independent of salivary flow and thus effectively correct for a loss of saliva water.

However, the use of total protein has been criticised because certain salivary proteins are sensitive to physiological conditions and may influence total protein secretion rate (Gleeson, 2000b). Increases in protein concentration have been found (Hübner-Wozniak et al., 1997; Ljungberg et al., 1997) even when s-IgA concentration remained unaffected (Walsh et al., 1999). Several authors have claimed that the ratio of s-IgA to protein may be misleading as the saliva flow rate alters during exercise, leading to a higher output of total protein without necessarily a parallel change in the output of s-IgA (Blannin et al., 1998; Gleeson, 2000a; Miletic et al., 1996).

Using the IgA:albumin ratio would be preferable because albumin is passively transported into saliva from plasma, where it is under homeostatic control. However, even albumin concentration or transportation can be affected by several factors such as plasma volume, postural change, pregnancy, congestive heart failure, severe malnutrition and liver failure.

Another way to correct for loss of saliva water is to express s-IgA relative to osmolality, which increases in proportion to the fall in saliva flow rate (Blannin et al., 1998). Osmolality is a measure of the total dissolved particle concentration. Because inorganic electrolytes rather than proteins (< 1%) contribute most to the osmolality of saliva, the overall concentration of solutes (or osmolality) should not be affected by most physiological conditions (Blannin et al., 1998). However, the measurement of osmolality requires additional equipment.

The protective effect of s-IgA in the upper respiratory tract depends on its secretion rate (Mackinnon and Hooper, 1994), which determines the total amount of s-IgA available at the mucosal surface. Therefore, the s-IgA secretion rate (expressed in $\mu g \cdot min^{-1}$) would appear to be a good way to express s-IgA. A drawback of this method is that it requires the correct assessment of total saliva flow rate without any losses (e.g. due to swallowing). It also requires exact timing of the period of sampling. Poor compliance of subjects adversely affects this measurement.

Unfortunately the s-IgA measurement chosen can alter the interpretation of experimental results. This is illustrated by the results from a pilot study (Pacqué et al., 2002). Fifteen males and six females completed two laboratory stress tests (walking at $5km\,h^{-1}$). Albumin and s-IgA were measured in saliva samples collected immediately upon completion of each test and at timed intervals up to 24 hours later. For each subject the results from both tests were averaged. Although there were significant

changes in each measure in the first hour post exercise, the trend was for an increase in s-IgA concentration, an immediate decrease in secretion rate followed by an increase, and a suppression of IgA:albumin for at least one hour.

1.3.7 Diurnal variation of salivary IgA

Dawes (1972) established that the flow rate and concentration of several components (IgA was not measured) of unstimulated whole saliva show a significant circadian rhythm. It was concluded that "*the presence of circadian rhythms in salivary flow rate and composition must influence the concept of normal values and in any study on saliva the time of day of sampling could have an important bearing on the results*" (Dawes, 1972). For this reason in any comparative study, saliva samples should ideally be collected at the same time of day. The possible presence of a diurnal variation has, however, not always been considered in the design or interpretation of past research on the effects of exercise or stress on s-IgA.

Findings on the magnitude and pattern of a possible diurnal variation are equivocal. Several authors have reported that there is an early morning high, followed by a fall to a lower stable base in the afternoon (Gleeson et al., 2001; Hucklebridge et al., 1998; Richter et al., 1980; Walsh et al., 2002). However, no clear pattern has emerged as not all investigators have measured s-IgA at the same times of day. For instance, in one study (Richter et al., 1980), s-IgA concentration was highest at 5:00, whereas in others it was highest at 8:00 (Gleeson et al., 2001) or at the time of awakening (mean awakening time was 7:30, range 6:00-10:30) (Hucklebridge et al., 1998). Others observed no differences in s-IgA concentration between mid-morning and mid-afternoon (Gleeson et al., 1990). Dimitriou et al. (2002) found significantly higher s-IgA concentrations in samples taken at 6:00, but secretion rate was significantly higher at 18:00. Butler et al. (1990) found some fluctuations in s-IgA concentration and secretion rate but commented that these were more characteristic of the subjects than the time of sampling. Miletic et al. (1996) found significantly higher morning s-IgA secretion rates compared with the afternoon in 20-30 years old subjects but not in 60-80 years old subjects. Based on these findings there is some evidence that s-IgA concentration and secretion rate exhibit a high early in the morning, followed by a decrease leading to a plateau from midday until evening. However, the evidence is not conclusive.

Several mechanisms have been proposed to explain a possible morning high. A first theory is that, as s-IgA secretion is influenced by sympathetic nervous system

activity, s-IgA secretion increases when a subject is relaxed (as when asleep) (Green and Green, 1987) and suppressed when a subjects is stressed (Jemmott et al., 1983). A second theory is that bright light exposure during the daytime can result in an increase in nocturnal melatonin levels due to a relaxation of the sympathetic nervous system, resulting in an elevated s-IgA secretion (Park and Tokura, 1999). A decrease in melatonin concentration in the morning would then result in a decrease on s-IgA. The mechanism by which melatonin exerts its stimulating effects on s-IgA is at present unknown. An attractive third theory is that the morning fall in s-IgA concentration and secretion rate is due to the acute stress caused by the awakening process and is negatively associated with the increase in cortisol levels observed over the same period (Hucklebridge et al., 1998).

A preliminary study conducted by the present author, found no evidence to confirm the existence of a diurnal pattern (Pacqué et al., 2002). There was a trend for higher early morning values (after waking), reasonably stable values during the day and a second smaller peak late in the evening. However, the data showed a wide scatter of results for time of day and a repeated measures analysis of variance revealed no significant effect of time. The number of subjects in this study was limited ($N = 19$).

The effect of fasting or eating on the diurnal variation of s-IgA is not clear. In one study, s-IgA concentration was found to be higher in fasting samples (range 25-600 $mg{\cdot}L^{-1}$) than in post-prandial samples (9-125 $mg\,L^{-1}$) (Gleeson et al., 1990) but no other studies have specifically investigated this.

1.3.8 Biological variation of salivary IgA

The literature indicates that there is a large within-subject (day-to-day and within day) and a large between-subject biological variation in s-IgA. Sometimes these variations seem to be of greater importance/magnitude than the changes caused by the experimental intervention. One study found that s-IgA concentrations within-subjects can vary by a factor of 2.53 between days (Gleeson et al., 1990), whereas in another study, there was a daily variation in s-IgA secretion rates in elderly (60-80 years old) but not in young subjects (20-30 years old) (Miletic et al., 1996). S-IgA concentration can differ 15-fold between individuals (Bratthall and Widerström, 1985). Individual differences in saliva flow rate, which influences s-IgA concentration, can be as great as 30-fold (Mayhall, 1975). The individual variability of s-IgA can be affected by the activity level and fitness of individuals as shown by Francis et al. (2005).

Our own observations have confirmed the existence of a large biological variation. Using a cohort of 21 adults, early morning (fasting) saliva samples were collected three times a week for eight weeks (Pacqué et al., 2002). The mean within-subject biological variation CV_I was 42.5% for s-IgA concentration and 44.9% for s-IgA secretion rate. The between-subject variation CV_G was 48.8% and 70.6% respectively and the index of individuality ($II = CV_I / CV_G$) was 0.87 and 0.64 respectively.

1.3.9 Reference interval of salivary IgA

One published reference interval for s-IgA concentration for Australian adults is 10-105 mg L^{-1} (non-fasting samples) and 20-500 mg L^{-1} (fasting samples) (Gleeson et al., 1990). Our own work, based on a group of 21 adults resulted in a fasting reference interval of 20-160 mg L^{-1} (Pacqué et al., 2002).

The large biological variation and low index of individuality (II) for measures of s-IgA cast some doubt on its value as a biomarker of stress if an individual's measurements are compared with a population-based reference interval. If the II is < 0.6 then an unacceptable high degree of individuality exists for the measurement and a population-based reference interval may not be very helpful for detecting significant change in repeated measurements for an individual (Solberg, 1994). Individuals may have an unusual measurement result for them, but still fall within the healthy reference interval or conversely, a measurement might be usual for an individual but fall outside the reference interval (Fraser, 1994). Therefore, using s-IgA in this way may have little value in predicting risk of ill health for an individual.

However, s-IgA may be useful to detect trends in the mucosal immunity of an individual if measurements can be compared against the individual's healthy baseline values. This would make it necessary to establish an individual's personal reference interval. In the case of athletes this might be problematic as physical activity levels seem to have an impact on baseline levels. For instance, baseline levels of s-IgA concentration of competitive cross-country skiers during heavy training were significantly lower than of sedentary controls (Tomasi et al., 1982) and baseline levels of collegiate swimmers during the competitive season were significantly lower than preseason values (Tharp and Barnes, 1990). From these studies, it appears that high volumes of intensive training depress baseline s-IgA levels. Many athletes have possibly been competing and training for a long time. Therefore, it is possible that low resting levels of s-IgA represent a cumulative effect over the years of training of

exercise-induced immunosuppression (Fricker et al., 1999). Because it is not easy to persuade athletes to interrupt their training even for a day, it may be difficult to determine their real "resting levels".

Finally, the possible presence of circadian rhythms makes it difficult to compare measurements with reference values. As the time of day that samples are taken may, have a bearing on results, it is advised that in any comparative study, saliva samples should ideally be collected at the same time of day.

1.4 Exercise and immune function

There is both anecdotal and epidemiological evidence, albeit rather weak, that regular moderate intensity exercise reduces the risk of infections by boosting the immune system and for instance is associated with a reduced vulnerability to upper respiratory tract infections (URTI) (Nieman et al., 1990b). Intense and repeated endurance exercise, on the other hand, may perturb or compromise several components of the immune system (e.g. natural killer cells, lymphokine-activated killer cells, leucocytes, lymphocytes, neutrophils, cytokines, etc). Many of the changes of immune function are seen in peripheral blood; however, there is no strong evidence that either total cell numbers or activity is changed in the body as a whole. Changes are generally transitory, usually returning to normal within hours of most types of exercise. A possible exception is very prolonged exercise, such as ultra marathon running, which may induce long lasting (24 hours or beyond) changes (Schobersberger et al., 2000).

It has been proposed that after exercise there is an "open window" period of impaired immunity during which there is an increased susceptibility to infections by viruses and bacteria (Mackinnon et al., 1993b; Peters, 1997) and that the degree and duration of immunosuppression depends on the intensity and duration of the exercise (Pedersen et al., 1996). Although the open window concept is an attractive hypothesis, Nieman (2000) stated that there is no proof that the athletes showing the most extreme immunosuppression following heavy exertion are those that contract an infection during the following period. According to Pedersen et al. (1994) post exercise immunosuppression may actually confer a benefit: since severe physical stress is associated with tissue breakdown and presentation of new antigens to the immune system, autoimmune reactions could be avoided.

1.5 Salivary IgA and upper respiratory tract infections

Nieman (1994) postulated there is a J-shaped relationship between physical activity and susceptibility to upper respiratory tract infections (URTI) (Figure 2). He suggested that regular, moderate intensity physical activity reduces the risk of URTI. In a recent study by Ciloglu (2005), moderate intensity aerobic exercise was indeed associated with fewer episodes of URTI in healthy women. On the other hand, very high intensity or large amounts of exercise would increase the risk. According to this hypothesis, one would expect elite and endurance athletes to have a higher incidence of URTI than non-athletes or moderately exercising subjects. Roberts (1986) suggested that URTI is the most common infection in highly trained athletes and URTI have been cited as comprising on average 96-98% of "*all types of pathological cases*" in athletes (Levando et al., 1988). For instance, in a rare yearlong study of American football players, Fahlman and Engels (2005) found that a season of training resulted in an increase in the incidence of URTI. Pyne (1999) suggested that URTI are the main cause of missed training in elite athletes and may have a deleterious effect on an athlete's ability to compete. According to Gleeson (2000c) the period of highest vulnerability often coincides with the intense training undertaken immediately prior to or during a competition and may not follow the normal seasonal patterns observed in the general community. The J-shaped hypothesis is not universally accepted. Shephard (2000) warned that because it was largely built on a composite of results drawn from small and disparate studies it needs further substantiation.

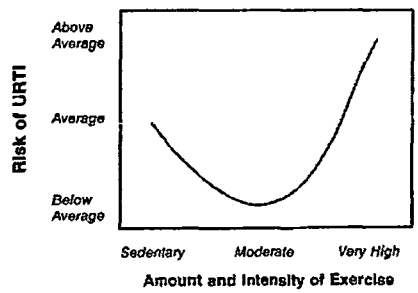


Figure 2: *"J"-shaped model of the relationship between amount of exercise and risk of URTI. From Nieman (1994), page 129. Reproduced with permission from Lippincot, Williams & Wikins.*

Secretory IgA is the body's first line of defence against invasion from certain pathogens. It is possible that there is a critical concentration of s-IgA required to provide adequate protection from URTI (Gleeson et al., 1999b) and that a lack of s-IgA or the inability to produce specific IgA antibodies can lead to an increased susceptibility

to URTI. Although according to Gleeson et al. (1999a) the precise association between exercise, URTI and immunosuppression remains unclear, transient changes in s-IgA levels or low resting s-IgA levels induced by exercise could contribute to the increased susceptibility of infection in athletes (Blannin et al., 1998; Mackinnon and Hooper, 1994; Tomasi et al., 1982). Nevertheless, it is yet to be unequivocally demonstrated that depressed levels of s-IgA predispose an individual to URTI.

For ethical reasons, experiments attempting to determine the influence of physical activity on the susceptibility of humans to instilled infection are unlikely to be undertaken. Epidemiological data are much more readily available. Findings suggest there is a dose-response relationship between the volume of physical activity and the likelihood of respiratory symptoms. In young adults, moderate exercise seemed to have little influence on the risk of URTI (Schouten et al., 1988). On the other hand, there is a strong consensus that very heavy exercise and/or training increases the prevalence and/or the persistence of respiratory symptoms (Shephard and Shek, 1999a). As early as the late 1970's increased levels of perceived URTI were reported in conditioned athletes (Douglas and Hanson, 1987). The first report of a direct association between URTI and s-IgA in athletes during periods of maximum intensity training came from a study of Russian Olympic athletes (Levando et al., 1988). Other studies have found temporal relationships between exercise, URTI, and possibly s-IgA, in athletes involved in various sports such as swimming (Gleeson et al., 1999a), squash (Mackinnon et al., 1992), hockey (Mackinnon et al., 1993b), and (ultra)marathon running (Nieman et al., 1990a; Peters and Bateman, 1983). In other studies, however, such temporal associations were not found. For instance, no association was found between the incidence of URTI and s-IgA concentration in a group of swimmers over a 12-week training period (Gleeson et al., 2000b), in a group of elite rowers (Nehlsen-Cannarella et al., 2000), and in a group of exercising post-menopausal Turkish women (Ciloglu, 2005). In a recent study on the incidence of URTI in French soldiers during four weeks of commando training Tiollier et al. (2005) found no relation between URTI and s-IgA.

The methodology, and therefore the conclusions of some of the studies that looked at the association between exercise and URTI can be criticised. Some studies possibly had a number of subjects that was too small to detect a significant effect. The subjects in many studies are only representative for a particular sub-population (e.g. elite swimmers) and findings can thus not be generalised. Some studies compared highly active with less-active individuals, without regard for differences in lifestyle, and many studies do not report the fitness level of their subjects at all. The diagnosis and

assessment of the severity and frequency of URTI ranged from subjective self-reporting (e.g. by questionnaire) to diagnosis by a medical practitioner. When questionnaires are used rather than clinical examination, the findings can be questionable for several reasons. There is the danger that return rates are low, that URTI prevalence is over- or under-reported, or that symptoms are misinterpreted. The aetiology of URTI depends on many factors other than the participation in strenuous exercise. The likelihood of contact with other infected people (e.g. spouse, children), the frequency of exposure to pathogens, gender, age, season, exposure to extremes of heat and cold, overall levels of (psychological) stress, overall levels of health, sleep deprivation, nutritional status and the use of dietary supplements, and an individual's ability to elicit specific immune and non-immune responses are a few factors that can influence the prevalence of URTI (McDowell et al., 1991; Shephard, 2000). Consequently, epidemiological data may not always provide a valid measure of prevalence of URTI.

1.6 Salivary IgA as a biomarker

The Australian Defence Force (ADF) 2000 Health Status Report suggested that the high usage of medications for treating coughs, colds and infections indicates that URTI is common within the ADF (Department of Defence, 2000). The susceptibility of soldiers to URTI has also been observed in several field studies. A 21-day Royal Australian Air Force survival course in northern Australia (Townsville) resulted in significantly reduced s-IgA levels which were associated with the incidence of URTI (Carins and Booth, 2002). A high incidence of URTI was also observed in 482 male soldiers engaged in heavy special warfare training (Linenger et al., 1993), in 477 Navy Special Forces trainees during 2 weeks of intense training (Gray et al., 2001b), and in 21 male cadets during commando training (Tiollier et al., 2005). In this last study the incidence of URTI was not related to s-IgA.

The danger of encountering unfamiliar viruses when soldiers (or athletes) congregate in groups for an extended period of time (e.g. at training camps) is real. The presence of URTI in athletes or soldiers during periods of intense training and competitions is of particular concern because URTI can decrease their capability to perform optimally. Early identification of individuals at risk of developing an URTI would allow training to be altered before performance (or operational readiness) deteriorates. Research is now directed towards identifying markers that could be used to this effect. Much attention has focused on s-IgA as it is the only immune parameter so far to be directly associated with the appearance of URTI (Carins and Booth, 2002; Gleeson et al., 1999b;

Mackinnon, 2000; Mackinnon et al., 1991; McKenzie, 1999). It is hoped that monitoring s-IgA levels at regular intervals could provide an indirect method for the assessment of immune status and to determine the risk of infection. S-IgA may also be a marker to detect over training syndrome (OTS) as this condition has been associated with high rates of URTI (Mackinnon and Hooper, 1994; McKenzie, 1999; Shephard and Shek, 1998).

Despite saliva sampling appearing to be convenient for field-based work, some caution is warranted with the use of this methodology. In an excellent recent review of the exercise immunology literature, Gleeson and co-workers summarised the limitations of the method (Gleeson et al., 2004). For example, the conditions of saliva collection can affect the measured IgA concentration (e.g. the source of the saliva; whether flow rate was stimulated or not; "fasting" or "non-fasting" state of subjects); careful handling of saliva samples is required during the transport, storage, and the analytical phase of experiments since s-IgA degrades over time; there is no uniformity in the analytical methods used to determine s-IgA concentration (instrumentation, technique, analytes); there is no sensitive and practical screening test for s-IgA that can be implemented in normal training; and there is no consensus about the best way to express s-IgA levels. Furthermore, s-IgA has a considerable biological variation and may be also subject to diurnal variation. These issues are addressed in Chapter 2.

A decrease in s-IgA has not been proven to be a valid indicator of the risk of URTI or excessive training (Shephard and Shek, 1998). There is as yet no clear understanding of the dose-response effect of intensity and duration of physical activity on s-IgA and the exact concentration (or secretion rate) at which this immune factor becomes predictive is still unknown (Nieman, 1994). It is not well understood how rapidly s-IgA levels return to baseline after a period of suppression in response to a stressor. Resting s-IgA measurements and the response to exercise may depend on factors such as fitness and activity levels and environmental conditions. Finally there is little information on the relationship between s-IgA and other biomarkers of immune function or OTS. These issues are addressed in the experiments described in Chapters 3, 4 and 5.

1.7 Salivary IgA and exercise

Tomasi et al. (1982) were the first to publish research on the effects of exercise on s-IgA. Compared with age-matched controls, they found significantly lower s-IgA concentration in cross country skiers before a race. S-IgA concentrations decreased

further following the competition. The authors speculated that a temporary antibody deficiency on the mucosal surface might lead to a susceptibility to acquiring viral and bacterial infections, especially immediately after strenuous exercise.

Since this initial study, various investigations have looked at the effects of exercise on s-IgA in a diverse range of individual and team sports, with athletes of a wide variety of age, fitness level, experience and training background. Studies of elite athletes have found transient decreases of 5-65% in s-IgA levels following intense endurance exercise in swimmers (Tharp and Barnes, 1990), distance runners (Cameron and Priddle, 1990), kayakers (Mackinnon et al., 1993a), triathletes (Steerenberg et al., 1997), cyclists (Mackinnon et al., 1989), and rowers (Nehlsen-Cannarella et al., 2000). In contrast, increases in s-IgA concentration, secretion rate and/or ratio to osmolality were reported in recreational athletes after maximal intensity exercise (Schouten et al., 1988), in marathon runners after a race (Ljungberg et al., 1997), in subjects of mixed physical fitness after ergometer cycling (Blannin et al., 1998) and in moderately exercising walkers and joggers (Buckwalter et al., 1996). Finally, s-IgA secretion rate did not change as a result of high-intensity intermittent ergometer cycling in well trained subjects (Walsh et al., 1999) or treadmill running in recreational and competitive runners (Mackinnon and Hooper, 1994).

The variety of responses in these reports may be explained in various ways, including major differences in their experimental design. For instance, the fitness level of subjects, the level of competition, the frequency, intensity and duration of exercise, and the metabolic energy source (aerobic versus anaerobic), can affect the s-IgA response to exercise (Gleeson et al., 2004; Hoffman-Goetz and Pedersen, 1994; Mackinnon, 1996; Nieman, 2000; Nieman and Nehlsen-Cannarella, 1991; Pedersen et al., 1994; Shephard, 2000). The disparity in findings may also arise from differences in the analytical methods. The non-uniformity of the s-IgA method and the differences in the interpretation of results, depending on the way in which s-IgA is expressed, are the biggest barrier to understanding the literature and determining the usefulness of s-IgA as a marker. Comparative analysis of published results is not valid unless the authors were measuring s-IgA in the same way. The table in Appendix A illustrates some of the differences in the methodology used (e.g. saliva source, collection method, assay methodology, antisera used and whether referenced to the international standard, and s-IgA measure reported.

1.7.1 Intensity and duration of exercise

The role of the intensity of physical activity on the s-IgA response remains unclear. Several studies, both under standardised laboratory conditions and under field conditions, have failed to show that changes in s-IgA in subjects of mixed physical fitness were related to exercise intensities between 50-100% of VO_{2max} (Blannin et al., 1998; Mackinnon and Hooper, 1994; McDowell et al., 1991; Walsh et al., 1999). Other studies, however, found that s-IgA levels were lowest after heavy workouts or during periods of peak training intensity (Dorrington et al., 2003; Levando et al., 1988; Mackinnon et al., 1993a; Tharp and Barnes, 1990).

The effect of the duration of exercise seems to be of secondary importance. Decreased levels of s-IgA have been observed after intense prolonged exercise such as several hours of competitive cross-country skiing (Tomasi et al., 1982) and ultra marathon running (Peters and Bateman, 1983), but also after intense exercise of short and very short duration. Changes in s-IgA were found to be similar following 15, 30 and 45 minutes of treadmill running at 60% of VO_{2max} (McDowell et al., 1991) and decreases of s-IgA secretion rate were observed after five 60-second bouts of supra-maximal interval exercise separated by 5 min rest (Mackinnon and Jenkins, 1993).

The cautious conclusion could be that exercise intensity rather than duration is the more important factor determining changes in s-IgA and that high-intensity physical activity is more likely to cause mucosal immune suppression than more moderate exercise. However, it is not clear if immunosuppression occurs at a threshold intensity and if there is a consistent dose-response. Additional well-designed studies are required to clarify the sometimes divergent findings and to determine whether exercise influences mucosal immune parameters in a uniform manner at specific intensities and duration. So far there is no evidence that changes in s-IgA caused by exercise are directly related to fitness or activity levels.

1.7.2 Long term effect of exercise

It is possible that exercising on a frequent basis may have a cumulative (suppressant) effect on the resting s-IgA concentration or secretion rate and on its response to acute exercise. However, in contrast to the large number of studies on the acute effects of exercise, much less is known about the long-term effect of physical activity on mucosal immune function. In an excellent review of the practical applications of exercise immunology, Nieman (1997) concluded that this is largely due to the difficulties in separating fitness effects from the actual physical exercise and that

ideally, such a study would require a large group of test subjects plus a control group to be followed for a relatively long period (e.g. one year). Although such a study has not been conducted to date, a few small longitudinal and cross-sectional studies have attempted to determine whether years of athletic training elicited significant changes in mucosal immunity. Cross-sectional studies have compared highly trained athletes with recreational athletes or sedentary individuals and longitudinal studies have monitored long-term changes in mucosal immune responses in athletes undertaking intensive training programs.

Lower resting s-IgA concentrations were reported in elite cross-country skiers, compared with control subjects (Tomasi et al., 1982) and in Russian Olympic athletes (Levando et al., 1988). In contrast, the resting s-IgA concentration was higher in trained rowers than in non-athletes (Nehlsen-Cannarella et al., 2000), and in a group of elite swimmers the pre-exercise levels of s-IgA were significantly higher than in a moderately exercising control group (Gleeson et al., 1999b). There was no difference between resting s-IgA concentration in competitive cyclists and age matched untrained control subjects (Mackinnon et al., 1987). Differences between study design, for instance the particular sport, the athletes' fitness levels, and the amount of psychological stress they were under, can all contribute to these conflicting findings.

Several longitudinal studies have found evidence of a cumulative suppressant effect of regular exercise on s-IgA. S-IgA concentration declined over 2–3 weeks of intensive training in elite kayakers (Gleeson et al., 2000a; Mackinnon et al., 1993a). Mackinnon et al. (1993a) found that the greatest decline in the IgA secretion rate occurred after the most intense training session at the end of a week of intense training. While it is possible that this was due to the training intensity, it may also reflect the cumulative effect of prior loads. Although there were no changes in s-IgA concentration in wrestlers over a 16 day training camp, when related to salivary protein, significantly decreased values were observed towards the end of the camp (Hübner-Wozniak et al., 1998). S-IgA secretion rate decreased over three consecutive days of intense treadmill running in a group of distance runners (Mackinnon and Hooper, 1994) and s-IgA:protein ratio decreased progressively over 5 days of competition in female hockey players (Mackinnon et al., 1992).

Progressive declines in s-IgA concentration were reported in swimmers over both a 3-month (Tharp and Barnes, 1990) and a 7-month training season (Gleeson et al., 1999a). Although a major difference between these two studies was that whereas in the first study s-IgA levels partially recovered in the tapering period leading up to

competition, they continued to decline in the second. In her excellent book on exercise immunology, Mackinnon stated that it is possible that the taper was of insufficient length to overcome the long-term immunosuppression or that the psychological stress associated with the upcoming competition may have prevented the recovery of s-IgA despite the reduction in physical stress (Mackinnon, 1999a). Two similar studies of elite swimmers on the other hand showed either no change in resting s-IgA concentrations over a 6-month season (Mackinnon and Hooper, 1994) or a small but significant increase over a 12-week training program (Gleeson et al., 2000b).

These studies suggest that regular training may indeed have a cumulative suppressive effect on mucosal immunity, and that lower resting s-IgA concentration can occur within a few days of training. More extended periods (i.e. several months) of sustained high intensity training may be required for persistent immunosuppression to occur.

1.7.3 Recovery after exercise

The rate at which s-IgA returns to pre-exercise or resting levels seems variable. In most studies, recovery occurred within one hour of the completion of exercise, as was the case for a marathon run (Ljungberg et al., 1997), a 10.5 km sub-maximal run (Cameron and Priddle, 1990) and a cycling ergometer exercise (Blannin et al., 1998). In this case the open window period of impaired immunity during which there is an increased susceptibility to infections (see section 1.4) is limited. However, other studies involving both elite and recreational athletes have documented slower recovery rates after exhaustive exercise. S-IgA levels remained depressed one hour after an incremental treadmill test to exhaustion in novice runners (McDowell et al., 1992a) and in competitive cyclists after a two-hour cycle ergometer tests (Mackinnon et al., 1987) and 90 min after two hours of rowing training (Nehlsen-Cannarella et al., 2000). S-IgA concentrations took 24 hours to return to pre-exercise levels after a bout of exhaustive cycling (Mackinnon et al., 1989).

1.7.4 Exercise in heat and humidity

Exercise in extreme heat and humidity could contribute considerably to the physiological stress of exercise as it introduces competing demands on the cardiovascular system: the need to supply oxygen to the working muscles and to the skin for cooling (Powers and Howley, 1997). It is essential for the body to maintain an internal temperature within narrow limits to prevent overheating independent of the

external environment. Despite the fact that elaborate temperature-regulating mechanisms are available, a combination of exercise and severe environmental conditions can impair or overwhelm homeostatic temperature regulation mechanisms and induce hyperthermia (Aoyagi et al., 1997 ; Brenner et al., 1995; Sutton, 1994). The heat liberated by the muscles during physical activity results in cutaneous vasodilation, increasing skin blood flow (Powers and Howley, 1997). Body heat is transported to the skin surface where it is eliminated largely through the evaporation of sweat. However, because homeostasis favours the maintenance of arterial blood pressure and circulation to the vital organs, blood is rerouted at the expense of thermoregulation when body core temperature reaches a critical value around 38°C (Aoyagi et al., 1997). This means that, under extreme climatic conditions and even when the exercise load remains constant, little further vasodilation is seen even if a further increase in core temperature occurs (Powers and Howley, 1997). If body temperature continues to go up, this will eventually lead to collapse (Sutton, 1994). As humidity increases, the body's major route of heat loss, evaporation, and therefore its ability for thermoregulation, diminishes (Powers and Howley, 1997). Evaporation accounts for about 25% of the heat loss at rest, but it is the most important means of heat loss during exercise (Nadel, 1988). As the relative humidity approaches 100%, this way of heat loss becomes increasingly reduced, eventually failing altogether. Although sweat is produced, it cannot evaporate and is lost to the cooling process (Powers and Howley, 1997).

There is little information available on immune responses to physical activity in a hot and humid environment. This is somewhat surprising for several reasons. According to Shephard (1998) the potential of exercise in hot and humid conditions to cause fatigue, exhaustion, and heat stress is well known. Second, Brenner et al. (1995) stated that physical activity in hot and humid environments is more likely to result in higher levels of hyperthermia which could contribute substantially to the immune system's response to exercise.

Some of the effects of hyperthermia on the immune system are similar to the changes caused by physical exercise (Hoffman-Goetz and Pedersen, 1994; Shephard and Shek, 1999b). For instance, both exercise and hyperthermia can result in changes in leukocyte counts and in increases in the blood concentration of stress hormones, such as adrenalin, noradrenalin and cortisol (Hoffman-Goetz and Pedersen, 1994; Kappel et al., 1997). It is therefore possible that some of the alleged exercise-induced changes in immune function may actually be temperature-induced (Brenner et al., 1995).

The effects of hyperthermia on immune function can be positive or negative (Shephard and Shek, 1999b). In their review on heat exposure and immune function, Brenner et al. (1995) concluded that in general, it seems that mild hyperthermia has little effect and that moderate hyperthermia enhances the immune system. They suggested that some forms of passive body heating, such as a sauna, allegedly augment immune function and have been advocated as a means of reducing the susceptibility to viral infections (Brenner et al., 1995). Shephard and Shek (1999b) state that the reason for any benefit is unknown and that it is unclear whether moderate hyperthermia has a direct enhancing effect on immune function or a deleterious effect on the viability of invading micro-organisms. Beyond the "therapeutic range" of hyperthermia, immune function becomes compromised (Brenner et al., 1995).

Given the general similarity in immune response to hyperthermia and vigorous exercise, one would anticipate an additive response to the combined stimuli. Shephard (1998) suggested that an intensity of physical activity or heat stress that is stimulatory or beneficial by itself could cause immunosuppression if the body is challenged by the two stimuli simultaneously. If vigorous exercise, severe enough to have an immunosuppressive effect by itself, is undertaken in a very hostile environment this effect is likely be more pronounced.

As early as 1982, it was suggested that studies were needed to "*determine the separate influences of ambient temperature versus exercise*" on mucosal antibodies (Tomasi et al., 1982). Nevertheless, little research has been done on the combined or separate effects of environmental stressors and exercise on s-IgA. A decline in s-IgA concentration has been reported as a result of occupational heat stress (Scheller et al., 1977) but, to the best of our knowledge, there are only three studies on the combined effects of heat, humidity and exercise on s-IgA. The findings of the first of these suggest that moderate intensity exercise, at a wide range of ambient temperatures, does not increase the susceptibility to URTI by decreasing s-IgA (Housh et al., 1991). Relative humidity (RH), which can contribute considerably to environmental stress in hot environments, was not a variable tested in this study. More recently, the s-IgA response to two hours of cycling at moderate intensity (63% of VO_{2max}) in a hot environment (30.3°C, 76% RH) was found to be no different from the same activity in temperate condition (20.4°C, 60% RH) (Laing et al., 2005). The third study investigated the combined effects of load carriage and environmental stress on s-IgA in Army reservists, aged 18 - 54 years (Pacqué et al., 2002). The thermal and cardiovascular strain experienced during a 2 hour march at 5 km hr^{-1} with a 20kg load in cool-dry (19°C; 51%

RH) and hot-humid (28°C; 80% RH) environments caused significant post-exercise changes in s-IgA concentration and s-IgA secretion rate with each variable returning to pre-exercise levels by 2 h post-exercise. However, there was no difference between the effects of the two environments.

1.7.5 Mechanisms underlying exercise-induced changes in salivary IgA

In a major review of the literature, Mackinnon (1999a) concluded that the exact mechanisms for changes in s-IgA concentration and secretion rate caused by exercise are still not fully understood. Possible mechanisms include changes in the mucosal surface of the mouth (Tomasi et al., 1982), suppression of IgA secretion and/or SC-mediated transport across the mucosal epithelium, and reduced migration of IgA-secreting plasma cells to the oral mucosa (Mackinnon, 1996). It is likely that both the neural and endocrine systems are involved and that the effects of exercise may be mediated through the actions of stress hormones, cytokines and haematological factors (Housh et al., 1991; McDowell et al., 1992a). Moreover, it appears that a reduction of saliva flow rate accounts in part for this response (Fahlman et al., 2001; Mackinnon and Jenkins, 1993)

During exercise a combination of high respiratory flow rates and a switch from nose to mouth breathing causes progressive cooling and drying of the respiratory mucosa. It was therefore postulated that the decrease in s-IgA levels following a competitive cross-country ski race might have been due, in part, to "*a large inflow of cold air which would likely lower the temperature of the mucous membranes*" (Tomasi et al., 1982)). Since this is where the plasma cells reside that secrete s-IgA, the authors reasoned that cooling of the mucous membrane might alter or impair the function of the plasma cells thereby reducing antibody secretion. The fact that s-IgA concentration or secretion can also be reduced by exercise when conducted in the warm and humid environments, such as in a swimming pool, somewhat refutes the temperature argument (Tharp and Barnes, 1990).

It is possible that intense exercise alters the process of production and secretion of s-IgA by plasma cells or affects the migration of plasma cell precursors to the oral mucosa (Mackinnon et al., 1987). However, Hucklebridge et al. (1998) suggested that the fact that changes in s-IgA secretion occur rather rapidly possibly reflects changes of the trans-epithelial transport mechanism rather than regulation at the level of plasma cell production of IgA, a process requiring days rather than minutes. To reach the mucosal

surface, s-IgA must be transported from the basal to the luminal face of the epithelium by secretory component and this process is possibly influenced by exercise (Mackinnon, 1999a).

The decrease of s-IgA levels may be due to a decrease in saliva flow. It is, however, not clear which mechanisms are responsible for this (Mackinnon and Jenkins, 1993), as it is a complex process, involving input from the parasympathetic and the sympathetic nervous systems (Mackinnon, 1999a).

There is a close relationship between the immune and neuroendocrine systems (Nieman, 1994) - they produce and use many of the same signal molecules in the form of hormones, lymphokines, and monokines for communication and regulation. Exercise, especially when the intensity is high, stimulates the neuroendocrine system, causing an increase in the blood concentration of a number of stress hormones, such as adrenalin, noradrenalin, growth hormone, β-endorphin and cortisol (Hoffman-Goetz and Pedersen, 1994). Although salivary cortisol has also been shown to increase in response to strenuous exercise (OConnor and Corrigan, 1987) and emotional stress (Tharp and Barnes, 1990), cortisol does not appear to mediate changes in the secretory process of s-IgA with exercise (Hucklebridge et al., 1998; McDowell et al., 1992b).

1.8 The overtraining syndrome

Overtraining has been described as "*a combination of signs and symptoms which typically cause the sufferer to feel mentally fatigued in the absence of physical fatigue and causes deterioration of performance*" (Kent, 1994). Overtraining syndrome (OTS) is a complex clinical condition with numerous potential contributing factors that vary considerably among individuals (Kuipers and Keizer, 1988; Lehman et al., 1993). The term OTS is most often used to describe athletes suffering from prolonged fatigue and impaired athletic performance, following a period of heavy training or competition, resulting from an imbalance between training and recovery (Budgett, 1990). It may appear when training sessions are too intense and/or prolonged, or when the duration of recovery periods is too short (Kuipers and Keizer, 1988; Lehman et al., 1993). The prevalence of OTS varies by sport and is thought to be highest in endurance sports requiring high volumes of intense training (Mackinnon, 2000). OTS is more likely to develop when physical training is combined with other stresses such as psychological stress and inadequate nutrition (Chicharro et al., 1998a; Mackinnon, 2000). For instance, carbohydrate-poor diets or training programs that chronically deplete glycogen stores can lead to OTS (Budgett, 1990). An individual's susceptibility to OTS will

depend on factors such as their exercise capacity, recovery potential and ability to cope with stress.

1.8.1 Symptoms and markers of overtraining

Many symptoms have been associated with OTS (Adlercreutz et al., 1986; Budgett, 1990; Chicharro et al., 1998a; Fry et al., 1992; Mackinnon, 2000; McKenzie, 1999): persistent fatigue and insomnia; under performance, decreased aerobic capacity and decreased anaerobic power; a "loss of purpose", energy and competitive drive; feelings of helplessness and incompetence; being trapped in a routine; loss of libido; emotional lability, increased anxiety and depression, mood-state disturbances, irritability; loss of appetite, weight loss, diarrhoea; muscle soreness, excessive sweating, increased resting heart rate, increased resting blood pressure, increased maximal plasma lactate concentration, decreased serum ferritin; immune suppression; and hormonal changes. OTS has also been associated with a high frequency of URTI (Fitzgerald, 1991). However, other than persistent fatigue and a decrement in performance, few have been consistently documented and some are not supported by the research literature at all (Mackinnon, 2000). Many of these factors also appear as symptoms of other conditions or diseases (Budgett, 1990). For instance, OTS has several similarities with chronic fatigue syndrome and clinical depression (Derman et al., 1997). Therefore, individually, none of these factors is as yet considered a reliable and valid indicator of OTS. At this stage, OTS is still more easily detected by decreases in physical performance and alterations in mood state.

There is as yet no single biological indicator that has been identified as a reliable marker of OTS (McKenzie, 1999) and there is no accepted gold standard for its diagnosis (Budgett, 1990). Several indicators have been proposed (clinical, neuroendocrine, physiological, psychological or biochemical) but unfortunately most of these vary considerably among individuals (Kuipers and Keizer, 1988; Lehman et al., 1993). Following markers have identified and investigated as possible indicators of OTS:

- physiological variables (weight, physical performance);
- fatigue and mood states (Fry et al., 1994);
- haematological variables (leukocytes; neutrophils; lymphocytes; monocytes; neutrophil to lymphocyte ratio; haemoglobin) (Lehmann et al., 1997)

- biochemical variables (norepinephrine and epinephrine (Hooper et al., 1993); cytokines (Sharp and Koutedakis, 1992; Smith, 2000); lactate (Jeukendrup and Hesselink, 1994); C-reactive protein (Thomas et al., 2000); TNFα (Sharp and Koutedakis, 1992); ferritin (Gastmann et al., 1998); free testosterone (Hoogeveen and Zonderland, 1996); cortisol (Verde et al., 1992); FTCR (Budgett, 1990; Vervoorn et al., 1991)).

According to Budgett (1990) the most promising marker to date seems the serum free testosterone to cortisol ratio (FTCR) which represents a balance between anabolic and catabolic activity within the tissues. The FTCR is related to physical performance (Hakkinen et al., 1985; Mujika et al., 1996) and a condition of overtraining may exist in an athlete if the FTCR decreases by 30% as this indicates an increased catabolic state (Adlercreutz et al., 1986; Chicharro et al., 1998a). However, not all studies agree with this. Although decreases in FTCR of up to 50% were observed in a group of elite rowers during a nine month period leading up to the 1988 Olympic Games, this was attributed to inadequate recovery from intensive training rather than to overtraining (Vervoorn et al., 1991). And in a study of professional cyclists it was concluded that a decreased FTCR does not necessarily lead to performance decrements or overtraining (Hoogeveen and Zonderland, 1996).

1.8.2 Mechanism of overtraining

The mechanisms causing OTS have not been clearly identified and it is unlikely that the diverse symptoms can be explained by a single mechanism (Budgett, 1994; Mackinnon, 2000; McKenzie, 1999). Although neuroendocrine changes may underlie many of the effects and symptoms of OTS (Budgett, 1990; McKenzie, 1999), the effects of heavy physical exercise on the neuroendocrine system and its relationship to overtraining are as yet unclear. The physical stress of intense exercise training may cause elevated stress hormone levels that may ultimately lead to autonomic dysfunction (Mackinnon, 2000). One hypothesis suggests that increased cytokine production is implicated (Smith, 2000). Repetitive, high volume exercise with inadequate rest may cause micro-trauma to joints, muscles and connective tissue. According to Smith (2000) this could activate monocytes to produce and release inflammatory cytokines which would then initiate a 'whole-body' response, involving chronic systemic inflammation, suppressed immune function, and mood state changes.

1.8.3 Salivary IgA and overtraining

Severe immunosuppression may occur in athletes who do not allow their immune system to recover, but initiate a new bout of exercise while it is still compromised (Pedersen et al., 1996). It is therefore not surprising that the OTS has been associated with immunosuppression (Budgett, 1990), recurrent infections and a high rate of URTI in athletes (Fitzgerald, 1991). One possible reason for a correlation between OTS and an increase in URTI in elite athletes could be that in overtrained athletes the open window of opportunism for pathogens to cause an infection is longer and the degree of immunosuppression more pronounced.

There is some evidence that s-IgA is suppressed in athletes during prolonged periods of intense exercise training leading to OTS. Among 14 elite swimmers over a 6-month season, s-IgA levels were lower in overtrained swimmers compared with those who did not show signs of overtraining (Mackinnon and Hooper, 1994). However, the concept that OTS is associated with an increased risk of URTI is not universally supported and it has been suggested that the risk of URTI is elevated in athletes during periods of intense training, regardless of whether such training leads to symptoms of OTS (Mackinnon, 2000). Nevertheless, as there is possibly an increased risk of URTI among endurance athletes, and a dose–response relationship between training volume and incidence of URTI, it appears that both OTS and URTI could result from a common cause: excessive training with insufficient rest and possibly a lack of variety in training.

1.9 Salivary IgA, psychological stress and mood states

It is well established that the autonomic nervous system (ANS), and the immune system are intimately linked. There is considerable research evidence that the immune system can be depressed following high levels of stress, emotional trauma, and by certain personality attributes (Jemmott et al., 1983). Neuroendocrine factors, such as stress hormones (e.g. catecholamines and cortisol), are thought to mediate at least some of the stress-induced changes in immunity (Kugler et al., 1996; Mackinnon et al., 1989). As saliva secretion is also regulated by the ANS (Chicharro et al., 1998b), it is hardly surprising that salivary immunoglobulin levels are down-regulated by chronic psychological stress (Hucklebridge et al., 1998) and that, similar to intense exercise, stress has been associated with an increased susceptibility to infections (Graham et al., 1988).

Significant associations have been found between chronic psychological stress and s-IgA (Graham et al., 1988; Jemmott et al., 1983). Transient decreases in s-IgA

secretion rates have been reported in a variety of stressful situations, for instance in dental students with increasing stress levels over an academic year leading up to examinations (Jemmott et al., 1983). Transient increases in s-IgA concentration were also found in soccer coaches under stress during matches (Kugler et al., 1996) and progressive suppression of s-IgA concentration and secretion rate was observed in subjects exposed to examination stress (Deinzer and Schuller, 1998). On the other hand, there were no significant changes in s-IgA in 26 subjects despite elevated anxiety levels before an important interview (Somer et al., 1993). Negative mood states other than anxiety (e.g. depression) have also been associated with decreased s-IgA concentrations (Graham et al., 1988; Jemmott et al., 1983; Mackinnon, 1999b). Others have found positive associations of s-IgA concentrations with positive mood states (Hucklebridge et al., 2000; Stone et al., 1987). Conversely, one study found mood states in everyday life to be unrelated to s-IgA concentration (Kugler et al., 1992).

The association between exercise induced mucosal immunosuppression and stress in athletes is not clear. Mackinnon and Hooper (1994) assumed that it is likely that the psychological stress of training and competing at the elite level can have an additive effect to that of physical stress on mucosal immune function. Others found no correlation between anxiety scores and s-IgA concentrations over a 7-month training season in swimmers (Gleeson et al., 1999b). So far there are no convincing arguments for either stress- or mood-induced immunosuppression or enhancement.

The widely used Profile of Mood States (POMS) questionnaire (Mc Nair et al., 1971) could be useful in identifying athletes who are immunosupressed and possibly on the verge of overtraining (Morgan et al., 1987). However, so far neither a consistent nor a simple pattern of results has emerged from the few studies that have tried to investigate this. One study in swimmers after high-intensity training, found a significant association between the POMS tension-anxiety subscale and changes in s-IgA (Tharp and Barnes, 1990). Another study of elite swimmers, however, showed no such association (Gleeson et al., 1999b). Of course, it is possible that the POMS scale is not sensitive enough to measure the emotional states, which might suppress the immune system. Although some of these studies suggest that acute psychological stress produces a temporary decrease in s-IgA, a strong correlation between s-IgA and stress or anxiety caused by exercise has yet to be established.

1.10 Purpose of this study

Exercise can cause a transient suppression of s-IgA and training at an intense level over many years often results in a chronic suppression of s-IgA in athletes (Gleeson, 2000a; Gleeson et al., 2000b; Steerenberg et al., 1997). There is also evidence that high levels of psychological stress are associated with a decrease in s-IgA levels and an increased susceptibility to infections (Graham et al., 1988; Jemmott et al., 1983). Therefore, it is not surprising that it has been demonstrated that soldiers, exposed to physiologically and psychologically stressful situations, are susceptible to mucosal immunosuppression and an increased risk of URTI (Booth et al., 2003; Brenner et al., 2000; Gomez-Merino et al., 2003; Gomez-Merino et al., 2005; Gray et al., 2001a).

Soldiers can also be susceptible to OTS. Army units are often composed of recruits who upon enlistment are not necessarily very fit. Thus, strenuous physical activity might be especially stressful for them. For instance, about one third of army recruits were classified as overtrained after an eight week physically demanding training program (Chicharro et al., 1998a). Symptoms of overtraining have also been documented during military training in the USA (Kramer et al., 1997) and Norway (Opstad, 2001).

A simple diagnostic test to assess the effect of military training and to monitor soldiers for an increased vulnerability to illness or early states of OTS would be very useful. With the assistance of such a biomarker, practices such as altered work-rest cycles, improved diet and psychological techniques could be used to minimise the impact of training on the health of military personnel. S-IgA could be useful for this purpose but because there are still several limitations, further work seemed warranted to investigate this marker.

The purpose of the work described in Chapter 2 was to address several methodological issues. Our preliminary work on s-IgA raised some concerns regarding its reliability as a biomarker (Pacqué et al., 2002). The main weaknesses identified were the possible effects of diurnal variation of s-IgA, its large biological variation, the way s-IgA is expressed, the standardisation of the s-IgA assay, and finally the collection, handling and storage of saliva samples.

The main purpose of the experiment described in Chapter 3 was to test the methods developed for the collection, storage and transport of saliva samples under "field conditions". It also assessed the effect of an ultra-endurance exercise on mucosal and humoral immunity and if changes in s-IgA and serum IgA correlated with changes in the incidence of upper respiratory tract infections.

The purpose of the study described in Chapter 4 was to determine whether a typical military field activity such as load carriage would result in decreased mucosal immunity. The impact of heavy physical work on soldiers' immune function is of particular interest to the ADF since it could have a direct effect on the health of their personnel. An infection resulting from immunosuppression in soldiers is likely to reduce performance and operational readiness, just as URTI in athletes may have a negative effect on their ability to train and compete optimally (Pyne, 1999).

The purpose of the work described in Chapter 5 was to investigate if changes in s-IgA were related to the incidence of URTI over the course of a seven week recruit training course. During basic military training, recruits are subject to both physiological and psychological stress, which could lead to ill health and OTS. By seeking an association between s-IgA measures and other variables identified as possible indicators of OTS it was investigated if s-IgA could be a useful predictor of OTS.

Overall, although this study seems to be heavily weighted towards the military, it aimed to contribute to the understanding of s-IgA as a potential biomarker to assess mucosal immune function and as a predictor of ill health or excessive physiological strain and diminished performance, not only in soldiers but also in athletes and in the general population.

METHODOLOGICAL CONSIDERATIONS ASSOCIATED WITH THE MEASUREMENT OF SALIVARY IgA

2.1 Introduction

Differences in methods and procedures often make it difficult to compare the results of studies that have measured s-IgA. Therefore, the first aim of this study was to optimise and standardise a method of saliva collection and storage before any further work was undertaken. In the first part of this chapter, the results of work that address this need are presented and recommendations are made.

The collection of saliva samples has several advantages over serum. It is low risk, easily retrieved and can be collected as many times as needed. However, there are several methodological limitations and disadvantages that make the usefulness of s-IgA for health monitoring open to question. Most of these limitations are related to the way people operate, not necessarily limitations to the potential use of s-IgA:

- There is inadequate uniformity in the laboratory procedures used to measure s-IgA in saliva;
- All measures of s-IgA (concentration, secretion rate, ratio to albumin, ratio to total protein, ratio to osmolality) have limitations;
- The method of saliva collection can influence the s-IgA concentration. One has to consider for instance the source of the saliva, the equipment used and whether saliva flow was stimulated or not;
- Careful handling of saliva samples is required during transport, storage, and the analytical phase to avoid possible degradation of s-IgA over time. Although anecdotal evidence has shown that s-IgA remains stable at room temperatures for a short period, this has not been previously investigated methodically by our laboratory.

Commercially available immunoassays and a Dade Behring BN ProSpec nephelometer (Dade Behring, Marburg, Germany) were used for the determination of IgA and albumin concentration in saliva samples. The accuracy, precision and linearity of the assays were established and the analytical coefficients of variation for the methods were verified for various sample pre-dilutions. The interference of a preservative on the assays was investigated. The stability of s-IgA under various

conditions of storage, including the effects of freeze-thawing was tested. Finally, quality control procedures were set up.

It has been suggested that s-IgA concentration and secretion rate are subject to a diurnal variation (Hucklebridge et al., 1998; Richter et al., 1980). If this is correct, this should be taken into account when interpreting the effect of any intervention on s-IgA. It would be essential to know whether any changes in s-IgA were caused, for instance, by exercise or were merely manifestations of its normal diurnal variation. Unfortunately the possible presence of diurnal variation in s-IgA has not always been considered in the design or interpretation of past research. On the other hand, its existence has not been established beyond doubt. So far findings are inconsistent and there is no consensus about the magnitude and the exact pattern of possible diurnal variations. Although in general studies suggest that s-IgA concentration and secretion rate are high in the morning, followed by lower stable values from mid-morning until early evening, there is no conclusive evidence of a distinct pattern.

In most previous studies early morning saliva samples were obtained after an overnight fast. Therefore, it was postulated that the nutritional status of the subject might be of some importance. However, to the best of our knowledge no-one has determined if eating could affect the diurnal variation of s-IgA, i.e. if there is a possible post-prandial effect. Although in one study s-IgA concentration has been found to be significantly higher in fasting samples than in post-prandial samples, the post-prandial samples were taken several hours rather than immediately after the meal (Gleeson et al., 1990) and more research seemed warranted to investigate this.

The second aim of this study was therefore to further test the hypothesis that s-IgA is subject to diurnal variation; to investigate the effect of sustained fasting; and to determine the duration and magnitude of possible post-prandial changes in s-IgA. As s-IgA displays a large biological variation, this study investigated if there is a time of day when the variability in s-IgA is at it lowest and if so, if this would be the most appropriate time to determine s-IgA in a group of subjects.

To allow the interpretation of s-IgA measurements, a suitable reference interval is necessary. Although reference intervals for s-IgA concentration have been published previously, for instance by the DSTO (Pacqué, 2001) and by Gleeson et al. (1990), the final aim of the work described in this chapter was to establish a reference interval using the methodology established for this study.

2.2 Methods

Approval for the projects described in this and all further chapters of this PhD study was obtained from the Northern Tasmania Health & Medical Human Research Ethics Committee and the Australian Defence Human Research Ethics Committee (ADHREC) if military personnel were involved. The procedures of all projects conformed to the guidelines for human research ethics described in the Declaration of Helsinki (1989). For each project, subjects gave written informed consent after explanation of its nature, purpose and possible effects.

2.2.1 Subjects

To obtain the saliva samples needed to establish the methodology of this project and to investigate the stability of s-IgA, 24 subjects (ten females) were recruited from the staff of the School for Human Life Sciences at the University of Tasmania and of the Defence Science and Technology Organisation (DSTO) in Scottsdale.

To establish the diurnal and biological variation of s-IgA, and to investigate the effects of fasting, sixteen subjects (5 males, 11 females; aged 18 - 57), were recruited from the staff and students of the University of Tasmania and from the Launceston population.

The International Federation of Clinical Chemistry (IFCC) suggests that to establish a meaningful reference interval the minimum number of individuals required is 120 (Fraser, 2001). Therefore, to establish reference intervals for s-IgA, 134 subjects (93 males, 41 females; mean age 31, range 18 - 57) were recruited from staff and students of the University of Tasmania, DSTO staff and Australian Defence Force personnel.

Subjects had no self-reported signs or symptoms of an infection or infectious disease, were not on immunosuppressive drugs and were non-smokers.

2.2.2 Sample collection, preparation and storage

Saliva was collected in Salivette tubes with polyester swab (Sarstedt, Numbrecht, Germany). Sample collection was supervised by the investigator in most cases. After rinsing the mouth completely with water, and then waiting for one minute, saliva was collected over 2 minutes. This ensured a suitable volume of saliva for the various assays (0.5 mL). To avoid contamination with food or blood, saliva was not donated immediately after food consumption or within 45 minutes of tooth brushing, flossing or using a tooth pick. The polyester swab was tipped into the mouth without the fingers

touching it. So as not to stimulate saliva flow, the swab was kept still in the cheek or under the tongue. Talking, chewing or swallowing was not allowed. After two minutes, the saturated swab and any saliva that had accumulated in the mouth were expectorated into the Salivette. Care was taken not to swallow or spill any saliva. In order to measure saliva flow rate, saliva volume was determined by weight (± 0.01g) and the saliva collection timed accurately (± 1 sec). Saliva density was assumed to be 1.00 $g \cdot mL^{-1}$.

Saliva was recovered from the Salivettes by centrifugation for 4 min @ 4000 rpm (IEC Centra MP4R centrifuge; International Equipment Company, Needham Heights, Massachusetts, USA). Unless specified otherwise, samples were frozen at -80°C for later analysis (Econofreezer, Forma Scientific Inc., Marietta, Ohio, USA - or - Ultima Ultra Low Temperature Freezer, Revco Scientific Inc., Asheville, NC, USA). Samples collected at home were temporarily stored in a domestic freezer (-4°C) and delivered to the University within 48 hours.

For experiments requiring pooled saliva (e.g. for internal quality control samples), the saliva from nine subjects (4 females) was gently mixed before being aliquoted for storage. To investigate the requirement for a protease inhibitor, sodium azide (final concentration 0.2 $mg \cdot L^{-1}$) was added to some of these pooled samples.

To determine the stability of s-IgA, samples were stored at varying temperatures (28°C; 21°C, 4°C and -4°C) for periods ranging from 15 minutes to 15 days, with and without sodium azide. These temperatures were chosen to simulate typical field trial conditions. Another set of samples was stored at the more traditional -80°C for a period up to 90 weeks. To determine the effect of repeated freeze-thawing, multiple samples were frozen at -80°C, thawed, analysed and refrozen 6 times.

2.2.3 The commercial IgA and albumin immunoassays

The most common way of measuring salivary IgA concentration is by ELISA (see Appendix A). However, our laboratory used a Dade Behring BN ProSpec nephelometer with Dade Behring supplied consumables (diluent, reaction buffer, reagents, standards and controls) to measure s-IgA and albumin concentration. A similar method has been used by other teams (Aufricht et al., 1992; Tiollier et al., 2005). The IgA assay is a particle enhanced nephelometric immunoassay (PENIA). The commercial N Latex IgA test kit is made up of an IgA reagent (freeze-dried polystyrene particles coated with rabbit anti-human IgA); an IgA standard (a mixture of human sera) referenced to the International Federation of Clinical Chemists Certified Reference Material-470; an IgA supplementary reagent A (an aqueous solution of pyrrolidone and Tween); an IgA

supplementary reagent B (animal and human proteins as well as pyrrolidone); and an IgA control (a mixture of human sera). The Dade-Behring antisera detect both IgA subclasses (personal communication: Ralf Evelbauer, Dade-Behring Technical Support Group Plasma Proteins, 4 September 2002). For the salivary albumin assay an antiserum for human albumin, an SL protein standard, and various protein controls were used. Further information is available from the commercial assay kit inserts (Dade Behring, Marburg, Germany). All reagents, controls and standards were reconstituted with ultra-pure water from a Modulab Analytical - Research Grade UF/Polishing System (Continental Water Systems, San Antonio, TX, USA). The Dade Behring commercial assays for IgA in cerebro-spinal fluid (BN ProSpec protocol #62) and for albumin in urine (BN ProSpec protocol #27) were used. The addition of sodium azide (final concentration 0.2 $mg \cdot L^{-1}$) to saliva samples at various dilutions did not interfere with these assays (Table 1). Data on the reproducibility of the IgA and albumin assays is provided in section 2.3.2.

Table 1: IgA and albumin concentration in saliva samples at various dilutions, with and without sodium azide preservative (final concentration 0.2 $mg \cdot L^{-1}$)

Dilution factor	IgA concentration ($mg \cdot L^{-1}$) protocol # 62		Albumin concentration ($mg \cdot L^{-1}$) protocol # 27	
	Without Na Azide	With Na Azide	Without Na Azide	With Na Azide
1:5 (*N*=5)	25.7 ± 0.6	25.6 ± 1.1	42.9 ± 1.0	42.8 ± 0.4
1:25 (*N*=15)	97.8 ± 8.1	97.1 ± 6.0	44.3 ± 2.6	44.5 ± 2.0
1:50 (*N*=5)	104.8 ± 2.1	104.7 ± 1.5	41.6 ± 2.0	41.1 ± 4.8

All data are mean ± *SD*.

To establish the dynamic linear range of assay protocol #62, a series of dilutions were made of a serum control material to obtain known IgA concentrations in the range 4 - 103 $mg \cdot L^{-1}$ and measured values compared with expected values. A linear regression analysis showed that linearity of the assay was excellent ($P < 0.001$; $R^2 = 0.997$; $N = 25$; $y = 1.24x - 1.31 mg \cdot L^{-1}$). However, the measured values over that range differed significantly ($P = 0.007$) from expected values by 8.1 ± 10.0% (range -9.6% – 22.6%). For the range 4–10.3 $mg \cdot L^{-1}$ there was less bias ($P < 0.001$; $R^2 = 0.978$; $N = 13$; $y = 1.06x - 0.26$) and measured values did not differ significantly from expected values ($P = 0.384$; deviation 0.6 ± 6.6%; range -9.6% – 13.7%). It seems there was only a small systematic error at low concentrations, whereas it was quite large at high concentrations. This

indicated that the optimum range for the determination of IgA using assay #62 is 4 – 10.3 $mg \cdot L^{-1}$.

2.2.4 Salivary osmolality

Salivary osmolality, using the freezing point depression method, was measured on undiluted saliva samples with an Advanced Micro Osmometer, (Model 3300, Advanced Instruments Inc., Norwood, Massachusetts, USA). The reference solution and calibration standards were also provided by Advanced Instruments. Accuracy of the method was determined by repeatedly analysing a commercial control over seven days ($N = 34$). The average within run CV_A was 1.1% and did not exceed 1.8%. The between run CV_A was 1.2%. The intra and inter-assay precision were evaluated by repeatedly ($N = 26$) analysing an undiluted pooled sample on four separate days. The average within run CV_A was 1.9% and did not exceed 3.6%. The between run CV_A was 4.9%.

2.2.5 Diurnal variation

Seven saliva samples were obtained from sixteen fasting subjects in a single day (at 7:00, 9:00, 12:00, 14:00, 16:00, 18:00 and 20:30). Subjects were encouraged to have a late snack the evening before but then fasted from waking until after the 20:30 sample. At least 4 days later they provided another ten saliva samples: seven samples at times corresponding with those of the fasting day plus an additional sample at 7:30, 12:30 and 18:30 shortly after a non-standardised meal consumed at 7:00 (breakfast), 12:00 (lunch) and 18:00 (dinner). Subjects were not allowed to eat in between these meals. On both days, they were only allowed to drink water *ad libitum* and were specifically asked not to consume drinks containing alcohol or caffeine. Strenuous activity was avoided during both days. Samples were analysed in two batches (batch 1 = fasting day; batch 2 = non-fasting day) to reduce between run analytical error.

2.2.6 Reference interval

One hundred and thirty four subjects donated a single saliva sample. To minimize the possible effects of diurnal variation and eating, all samples were obtained at least two hours post-prandial and no early morning or late evening samples were used. These samples were analysed in 4 batches.

2.2.7 Quality control

The quality of the various assays was assessed by Levey-Jennings plots of in-house internal quality control (IQC) samples and commercial controls (Tietz, 1987). With a few exceptions (5 out of 65 for IgA and 2 out of 42 for albumin) commercial quality control values were within the manufacturers' specified control limits. Likewise, only a few albumin (3 out of 45) and osmolality (7 out of 69) measurements of IQC samples were outside their control limits, confirming the accuracy and precision of the methods. However, from week 68 onwards, the s-IgA concentration in the majority of IQC control samples was below the lower control limit. As the commercial controls analysed during this period remained within their control limits, it was unlikely that there was a problem with the accuracy of the method. This apparent downward shift in s-IgA concentration therefore suggested a breakdown of s-IgA after 68 weeks in samples stored at -80°C (see 2.3.3).

2.2.8 Statistical analysis

All statistical analyses for the experiments described in this and other chapters of this study were performed with SPSS (Statistical Package for the Social Sciences, versions 11.0 and 12.0, SPSS Inc, Chicago, USA). Data were tested for normality by visual inspection of histogram, by coefficients of skewness and kurtosis and by the Kolmogorov-Smirnov test. Data that were not distributed normally were either transformed by natural logarithm (ln) before further analysis by parametric tests or alternatively non-parametric tests were used. Descriptive statistics are presented as mean (*M*) and standard deviation (*SD*) or standard error of the mean (*SEM*). Significance was accepted at P values less than 0.05 for all statistical tests.

2.2.8.1 Methodology

To assess the effect of sodium azide as a preservative on the IgA and albumin assays, differences in means were compared by student t-test. Comparison between the expected and observed IgA concentrations to determine the linearity of the assay was performed by linear regression analysis. To compare the precision of the assays at various dilution factors, a repeated measures ANOVA was used. S-IgA stability over time at various storage conditions was assessed by repeated measures ANOVA and independent t-test.

2.2.8.2 Diurnal variation

Because the data sets were small, no outliers were removed. As data were not normally distributed, the non-parametric Wilcoxon signed ranks test was used to compare data sets. The possible effect of fasting was assessed by pair wise comparison of (1) all the fasting and non-fasting samples combined and (2) the seven corresponding time points of the fasting and the non-fasting days (i.e. the post-prandial samples of the non-fasting day were ignored). Changes across time on both days were assessed by pair wise comparison of the first sample with all subsequent samples (e.g. 7:00 and 9:00; 7:00 and 12:00; etc.) and by pair wise comparison of each pair of successive samples (e.g. 7:00 and 9:00; 9:00 and 12:00; etc.). To assess the effect of meals, pre-and post-prandial samples were compared (7:00 and 7:30; 12:00 and 12:30; 18:00 and 18:30).

2.2.8.3 Reference interval

Outliers in the data sets were removed using Reed's criterion (Fraser, 2001): extreme values were rejected if the difference between this value and the next highest (or lowest) was more than one-third of the absolute range of values. If several values were high (or low) compared to the others, then all of these values were rejected if Reed's criterion applied to the lowest (or highest) of them. As none of the distributions was normal, following non-parametric method recommended by the IFCC (Solberg, 1994) was used:

- The data were sorted in ascending order and ranked from 1 (minimum value) to n (maximum value).
- As the reference interval was defined as the central 95% interval bounded by the 2.5^{th} and 97.5^{th} percentiles of the distribution, the limits of the reference interval had rank numbers $0.025 * (n + 1)$ and $0.975 * (n + 1)$.
- The reference limits were the values that corresponded to these computed rank numbers. As these were not whole numbers, reference limits were found by interpolating between two appropriate values.

Differences between the means for the male and female subjects were assessed by independent t-test on natural log (ln) transformed data.

2.3 Results

2.3.1 Biological variation of salivary IgA

The biological variation for s-IgA was calculated using the samples from the subjects in the diurnal variation experiments. The average within-subject coefficient of variation CV_I, the between-subjects coefficient of variation CV_G and the index of individuality II ($II = CV_I/CV_G$) were calculated as suggested by Fraser (1994) and Fraser and Harris (1989). There was a large within and between-subject variation in all s-IgA measures (Figure 3; Table 2; see Appendix B for a table with the raw data and the CV_I for each individual). For some subjects the results on both days were very similar (e.g. subject 6), whereas for others they were dissimilar (e.g. subject 10). The CV_I and CV_G were somewhat lower in the fasting samples, suggesting that there was some effect of eating. In addition, the measures of s-IgA with the lowest variability tend to be the ratios and secretion rate. Table 3 shows the between-subject variability at different times of day. Again, the variability of s-IgA on the fasting day tends to be lower and the period of lowest variability tends to be between 12:00 and 18:00.

Table 2: Within-subject (CV_I) and between-subject (CV_G) coefficient of variation and index of individuality (II) for s-IgA measures on a fasting and a non-fasting day

s-IgA measure	Fasting day	Non-fasting day (all samples)	Non-fasting day (excluding post-prandial samples)
	Mean (range) of within-subject CV_I (in %)		
s-IgA concentration	73 (29-132)	64 (24-137)	55 (17-129)
s-IgA secretion rate	60 (19-139)	60 (26-98)	52 (22-94)
s-IgA:albumin ratio	45 (20-79)	57 (40-94)	44 (26-89)
s-IgA:osmolality ratio	59 (22-114)	55 (19-125)	44 (19-115)
	Between-subject CV_G (in %)		
s-IgA concentration	108	106	98
s-IgA secretion rate	95	114	109
s-IgA:albumin ratio	72	78	69
s-IgA:osmolality ratio	76	82	73
	Index of individuality (II)		
s-IgA concentration	0.68	0.60	0.56
s-IgA secretion rate	0.63	0.53	0.48
s-IgA:albumin ratio	0.63	0.73	0.64
s-IgA:osmolality ratio	0.78	0.67	0.60

Table 3: Between subject coefficient of variation (CV_G) for s-IgA measures at different times of day

s-IgA measures	Time of day									
	7.00	7.30	9.00	12.00	12.30	14.00	16.00	18.00	18.30	20.30
	Between subject coefficient CV_G (in %) on fasting day									
s-IgA concentration	88	n/a	126	44	n/a	82	67	74	n/a	50
s-IgA secretion rate	121	n/a	75	44	n/a	57	79	60	n/a	43
s-IgA:albumin ratio	72	n/a	71	83	n/a	54	65	58	n/a	60
s-IgA:osmolality ratio	73	n/a	83	43	n/a	71	66	63	n/a	43
	Between subject coefficient CV_G (in %) on non-fasting day									
s-IgA concentration	89	52	70	58	61	63	80	52	61	118
s-IgA secretion rate	125	78	88	56	76	94	95	73	56	125
s-IgA:albumin ratio	105	68	64	49	73	68	59	61	107	62
s-IgA:osmolality ratio	78	61	73	45	54	48	61	60	69	86

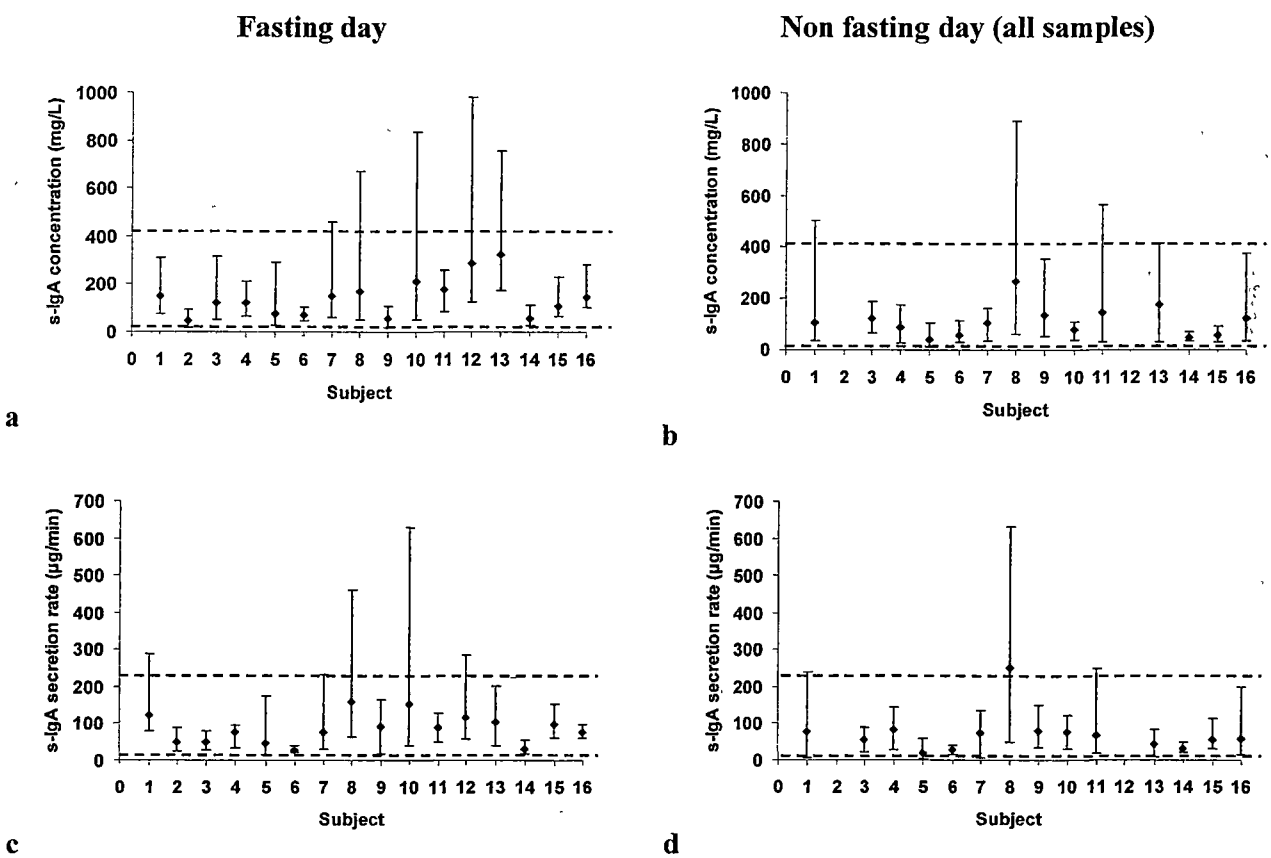


Figure 3: *The average and absolute range of s-IgA concentration (a & b) and s-IgA secretion rate (c & d) for each individual during a fasting and a non-fasting day show the variability of these measures. The dashed lines represent the reference interval calculated for a group of 134 subjects (see Table 10).*

2.3.2 Analytical variation of the assays

Analytical variation is expressed as the coefficient of variation of an assay. The accuracy of the IgA and albumin assays was determined by analysing multiple samples of commercial IgA ($N = 65$) and protein ($N = 42$) controls of known concentration. The between run analytical coefficients of variation (CV_A) for IgA was 4.9% and 8.3% for albumin. The precision of the two assays at various sample dilutions was determined by analysing five internal quality control (IQC) samples on up to six consecutive days. Albumin measurements were unaffected by the dilution factor but dilution had a significant effect on the s-IgA concentration. This can possibly be explained by the fact that the calibration curves established for albumin supported a much wider range of albumin concentrations that the calibration curve for s-IgA which supported only a narrow range. This would also mean that the linearity of the albumin assay was better over a wider range of albumin concentrations than that of the IgA assay (see section 2.4.1). The intra-assay and inter-assay analytical coefficients of variation CV_A are represented in Table 4. The best results were obtained with dilutions of 1:25 to 1:50 for s-IgA and 1:10 to 1:25 for albumin determinations.

Table 4: Analytical variation of s-IgA and albumin concentration in Internal Quality Control samples (pooled saliva) at various dilutions

Sample dilution	s-IgA concentration ($mg \cdot L^{-1}$)				Albumin concentration ($mg \cdot L^{-1}$)			
	Mean	*SD*	Intra assay CV_A	Inter assay CV_A	Mean	*SD*	Intra assay CV_A	Inter assay CV_A
1:5 ($N = 10$)	23.9	4.3	4.9%	18.0%	43.0	2.2	4.9%	5.2%
1:10 ($N = 5$)	65.6	5.5	8.4%	n/a	43.2	1.3	3.0%	n/a
1:25 ($N = 30$)	98.2	7.4	1.9%	7.5%	45.2	2.9	2.9%	6.5%
1:50 ($N = 30$)	105.9	5.5	1.8%	5.2%	44.0	4.1	6.3%	9.4%
1:71.5 ($N = 5$)	102.7	2.3	2.2%	n/a	42.8	2.3	5.3%	n/a

2.3.3 Salivary IgA stability

At various time points, specified in Table 5 & Table 7, two saliva samples were analysed at a 1:50 dilution, one with and one without the sodium azide preservative. As the s-IgA and albumin concentrations in these samples were identical, the results were averaged. A visual inspection of the results shows that there were some changes in both s-IgA and albumin concentration with time. At the higher temperatures (Table 5), most of these changes were smaller than the inter-assay coefficient of variation CV_A (Table

4). At 4°C and -4°C (Table 6), the s-IgA concentration increased considerably at first, then declined again. As only 2 samples were analysed at each time point, no statistical analysis was performed on these results.

Table 5: Stability of s-IgA and albumin at 28°C and 21°C

Time	s-IgA ($mg \cdot L^{-1}$) @ 28°C		s-IgA ($mg \cdot L^{-1}$) @ 21°C		Albumin ($mg \cdot L^{-1}$) @ 28°C		Albumin ($mg \cdot L^{-1}$) @ 21°C	
		% change		% change		% change		% change
Baseline	96.3	n/a	96.6	n/a	31.9	n/a	31.0	n/a
15 min	99.2	+3.0	93.6	-3.1	32.3	+1.3	32.0	+3.2
30 min	100.0	+3.8	95.9	-0.7	31.6	-0.9	32.3	+4.2
1 h	100.8	+4.7	95.6	-1.0	32.5	+1.9	32.0	+3.2
2 h	102.0	+5.9	100.1	+3.6	32.4	+1.6	32.0	+3.2
6 h	96.1	-0.2	100.2	+3.7	35.2	+10.3	33.1	+6.7
24 h	94.7	-1.7	88.4	-8.5	32.9	+3.1	30.5	-1.6
52.5 h	92.5	-3.9	84.7	-12.3	33.1	+3.8	30.4	-1.9

Table 6: Stability of s-IgA at 4°C and – 4°C

Time	s-IgA ($mg \cdot L^{-1}$) @ 4°C		s-IgA ($mg \cdot L^{-1}$) @ - 4°C	
		% change		% change
Baseline	73.7	n/a	71.0	n/a
3 h	86.9	+17.9	n/a	n/a
6 h	91.2	+23.7	n/a	n/a
24 h	101	+37.0	79.6	+12.1
32.5 h	100.2	+36.0	n/a	n/a
2 days	100.5	+36.4	n/a	n/a
6 days	79.6	+8.0	87.1	+22.7
15 days	77.7	+5.4	76.3	+7.5
198 days	n/a	n/a	74.9	+5.5

There were no changes in s-IgA concentration in samples stored at -80°C for the first 70 weeks (Table 7). S-IgA concentration was significantly lower in the period from week 71 to 80 and 91 to 90 than during weeks 21-30. Albumin concentration remained constant over the 90-week period.

Table 7: Stability of s-IgA and albumin concentration at -80°C

Period (week)	s-IgA ($mg \cdot L^{-1}$)	Albumin ($mg \cdot L^{-1}$)
21-30 ($N = 6$)	105.9 ± 5.5	44.0 ± 3.1
41-50 ($N = 4$)	122.3 ± 16.8	n/a
51-60 ($N = 10$)	100.7 ± 11.2	40.7 ± 5.4
61-70 ($N = 17$)	98.8 ± 9.5	43.9 ± 5.7
71-80 ($N = 8$)	80.2 ± 5.5*	46.2 ± 3.7
81-90 ($N = 10$)	87.4 ± 9.3*	44.1 ± 6.6

All values are mean ± *SD*. * significantly ($P < 0.05$) lower than weeks 21-70

A visual inspection of the data in Table 8 indicates that s-IgA and albumin concentration remained relatively unchanged after repeated freeze-thaw cycles and did not differ much from s-IgA and albumin concentration in control samples. No statistics were performed on these data as only two samples were analysed at each freeze-thaw cycle.

Table 8: Effect of freeze-thawing on s-IgA and albumin concentration

	Base	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
s-IgA ($mg \cdot L^{-1}$)	61.0	67.2	65.3	62.8	65.3	60.7	58.8
IgA control ($mg \cdot L^{-1}$)	61.0	67.8	69.8	69.2	66.6	62.9	66.2
Albumin ($mg \cdot L^{-1}$)	35.8	40.0	43.6	40.4	46.5	41.2	39.2
Albumin control ($mg \cdot L^{-1}$)	35.8	40.0	38.1	39.8	38.1	42.9	38.6

2.3.4 Reference interval

Table 9: Descriptive statistics for s-IgA in a group of 134 subjects

s-IgA measure	All subjects (n = 134)	Males (n = 93)	Females (n = 41)
Concentration ($mg \cdot L^{-1}$)	129.7 ± 102.6; 97.1; 13.7 – 483.0	151.5 ± 111.3; 125.5; 14.7 – 483.0	81.8 ± 56.5*; 64.7; 13.7 – 233.0
Secretion rate ($\mu g \cdot min^{-1}$)	70.9 ± 61.1; 50.7 6.6 – 313.7	84.1 ± 68.7; 71.7; 6.6 – 313.7	44.9 ± 28.7*; 36.7; 7.3 – 134.0
IgA:albumin	4.2 ± 3.9; 3.4; 0.3 – 27.1	4.4 ± 4.2; 3.5; 0.6 – 27.1	3.6 ± 3.3; 2.9; 0.3 – 17.2
s-IgA:osmolality ($mg \cdot mOsmol^{-1}$)	2.3 ± 1.5; 1.9 0.3 – 7.3	2.7 ± 1.6; 2.2; 0.6 – 7.3	1.6 ± 1.0*; 1.2; 0.3 – 3.6

All data are: mean ± SD; median; range. $P < 0.05$: * significantly different from males

To calculate a reference range, s-IgA was measured in a single sample donated by 134 subjects. Outliers were removed according to Reed's criterion (Fraser, 2001). The descriptive statistics are summarised in Table 9 and the frequency distribution of the s-IgA concentration and secretion rate data is shown in Figure 4. Calculated reference intervals are shown in Table 10.

Table 10: Reference interval for s-IgA measures for a group of 134 subjects

s-IgA measure	Lower limit	Upper limit
Concentration ($mg \cdot L^{-1}$)	15.9	414.5
Secretion rate ($\mu g \cdot min^{-1}$)	7.2	234.9
IgA:albumin	0.4	19.0
IgA:osmolality ratio ($mg \cdot mOsmol^{-1}$)	0.6	8.9

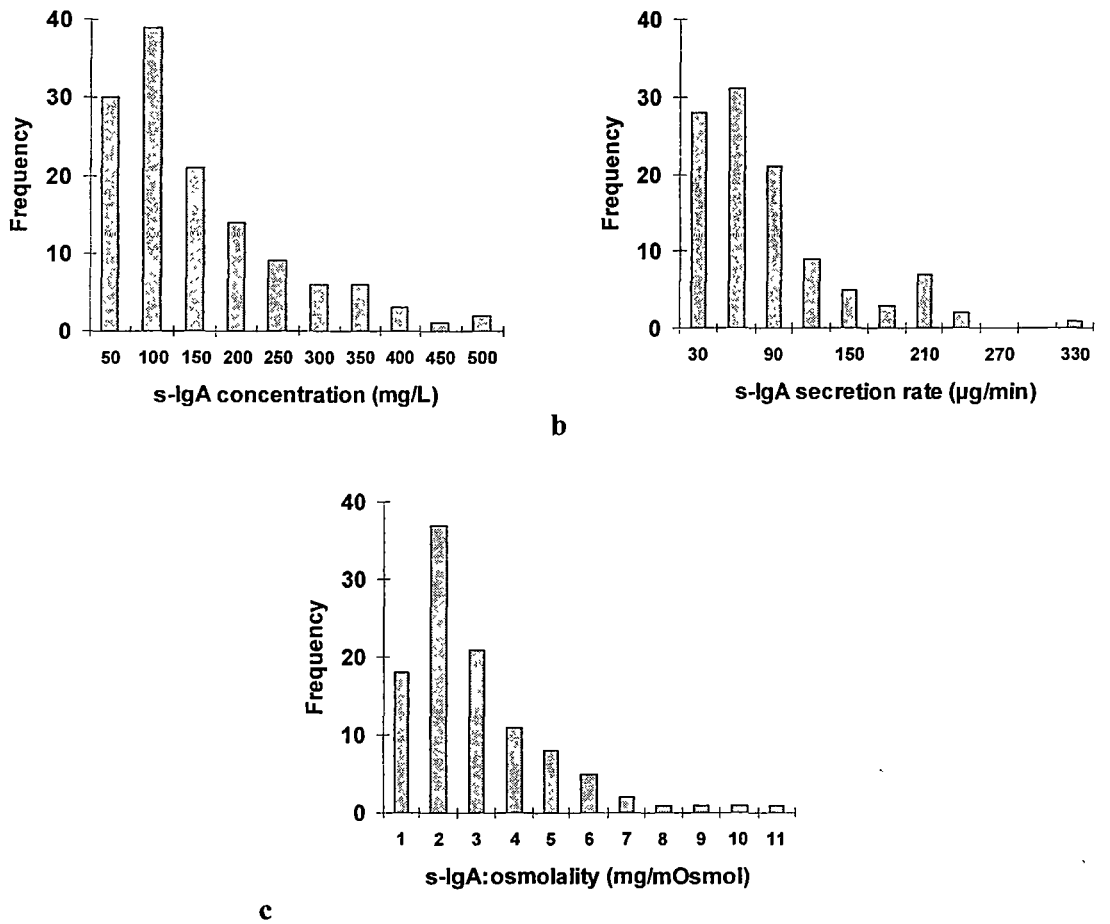


Figure 4: *The frequency distribution for s-IgA concentration (a), s-IgA secretion rate (b) and s-IgA:osmolality ratio (c) in a group of 134 subjects were all skewed.*

A stratified reference interval may be required if the difference between the means of potential subgroups is greater than 25% of the 95% reference interval for the entire group (Fraser, 2001). According to this criterion, stratification by gender was not necessary despite the fact that there were significant differences between the means of males and females for s-IgA concentration ($P < 0.001$), s-IgA secretion rate ($P = 0.006$), and s-IgA:osmolality ratio ($P < 0.001$).

2.3.5 Measures of s-IgA

So far in this chapter s-IgA levels have been expressed in four different ways: as a concentration, as a secretion rate, as a ratio to albumin, and as a ratio to osmolality. It is difficult to conclude which of the s-IgA measures is the best. As summarised in Chapter 1, each of them has disadvantages, some of which compromise the validity of the results. In the remainder of this chapter and in all subsequent chapters, only s-IgA secretion rate and s-IgA:osmolality ratio are reported. Theoretically, these two measures are the most valid and meaningful of the four measures. S-IgA secretion rate most accurately describes the amount of s-IgA available at the mucosal surface and by expressing s-IgA as a ratio to osmolality, changes in saliva flow and evaporation are corrected for. In addition, the present data indicate that the biological variation of the s-IgA:osmolality ratio is among the lowest. The s-IgA:osmolality ratio has the second lowest CV_I and CV_G for fasting samples and the second highest index of individuality (i.e. optimal) of all for samples.

2.3.6 Diurnal variation of salivary IgA

Comparing the seven common time points on a fasting and a non-fasting day, there was no consistency in the behaviour displayed by the s-IgA secretion rate and the s-IgA:osmolality ratio (Table 11 and Figure 5). There was a significant effect of time on s-IgA:osmolality during the fasting day ($F_{4,61} = 4.644$; $N = 16$; $P = 0.002$) and the non-fasting day ($F_{6,60} = 2.829$; $N = 12$; $P = 0.017$). On the fasting day, compared to the early morning, the s-IgA:osmolality ratio was lower at 14:00, 18:00 and 20:30 but not at 16:00. On the non-fasting day, when post-prandial effects were ignored, s-IgA:osmolality ratio was significantly lower at 12:00 and 18:00.

There was also a significant effect of time on s-IgA secretion rate on the non-fasting day ($F_{3,38} = 2.997$; $N = 12$; $P = 0.036$) but not on the fasting day ($F_{4,62} = 1.525$; $N = 16$; $P = 0.205$). S-IgA secretion rate remained unchanged throughout the fasting

day with the exception of a rise at 16:00. On the non-fasting day, when post-prandial effects were ignored, there were significant increases at 12:00 & 14:00.

There was no evidence for a morning high in s-IgA secretion rate which at no time was different from the 7.00 samples. S-IgA:osmolality ratio was lower than the early morning value at limited times only.

As illustrated by the graphs in Figure 5, s-IgA has a large biological variation. This makes it difficult to detect significant differences in small population groups.

2.3.6.1 The effect of a day of fasting on salivary IgA

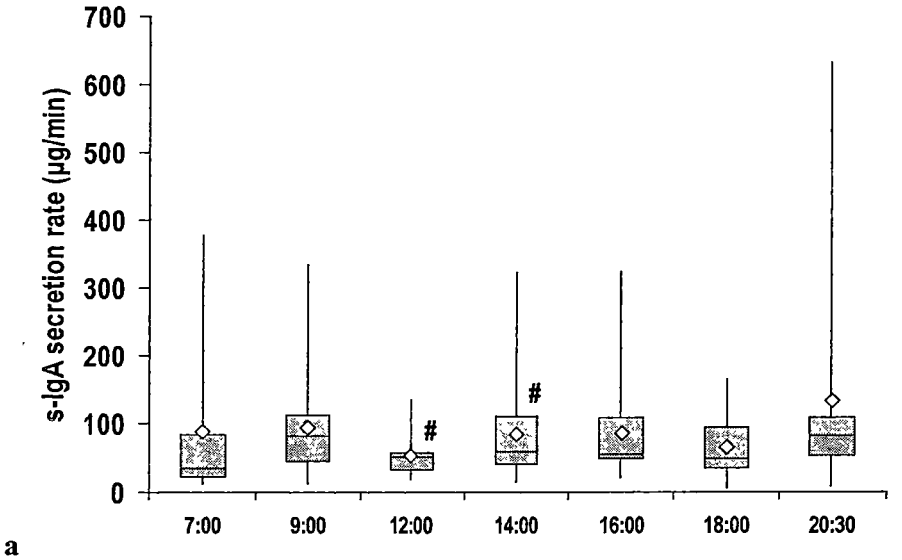
There were no differences between the s-IgA secretion rate and the s-IgA:osmolality ratio on the fasting and the non-fasting day. Neither were there any differences for s-IgA secretion rate at any individual time point. For s-IgA:osmolality ratio there was a significant difference between both days at 20:30 only ($P = 0.046$) (Table 11).

Table 11: Changes in s-IgA secretion rate and ratio to osmolality during a fasting and a non-fasting day

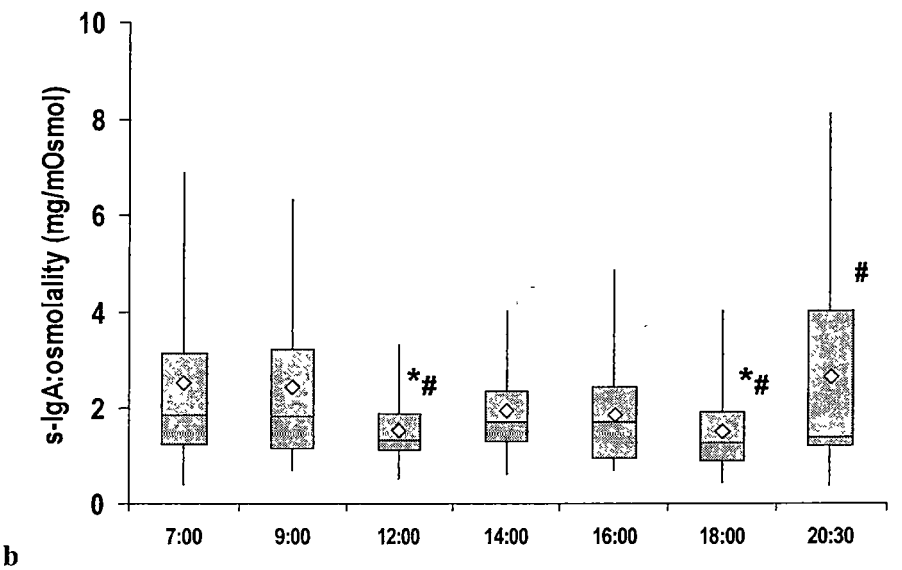
	Time of day						
	7.00	**9.00**	**12.00**	**14.00**	**16.00**	**18.00**	**20.30**
	s-IgA secretion rate (µg·min^{-1})						
fasting	77.1; 39.3 - 174.4	76.6; 49.2 - 106.2	74.6; 40.0 - 95.0	65.7; 26.8 - 105.0	71.0#; 25.5 - 118.1	78.9; 27.2 - 128.0	55.0; 39.9 - 83.
non-fasting	35.5; 21.6 - 149.7	81.4; 37.9 - 124.3	50.8#; 29.7 - 72.7	58.7#; 21.2 - 113.7	55.9; 26.4 - 111.8	49.4; 30.6 - 122.7	81.8; 40.3 - 198
	s-IgA:osmolality ratio (mg·mOsmol^{-1})						
fasting	2.3; 1.4 - 4.3	1.9; 1.4 - 4.1	1.6; 1.5 - 2.8	1.2*; 0.8 - 2.7	2.0; 1.0 - 3.1	1.4*#; 0.8 - 2.2	1.5†*; 0.8 - 1.8
non-fasting	1.9; 1.2 - 5.0	1.8; 1.1 - 3.5	1.4*#; 1.1 - 2.0	1.7; 1.3 - 2.9	1.7; 0.9 - 2.5	1.3*#; 0.9 - 2.2	1.4†#; 1.2 - 4.5

All data are median and 95% confidence limits for the median. The positions of the 95% confidence limits for the median in the sorted datasets were set as the position of the median plus and minus the square root of N with the lower limit rounded down and the upper limit rounded up to the nearest integer ((Snedecor and Cochran, 1980, page 137).

$P < 0.05$: * Significant difference with early morning sample (7:00); # Significant difference with the preceding sample; † Significant difference with corresponding sample on fasting/non-fasting day.



a



b

Figure 5: *Changes in s-IgA measured in 7 saliva samples during a normal (non-fasting) day (N = 16). There were some changes in s-IgA secretion rate (a) and s-IgA:osmolality ratio (b) throughout the day: * indicates a significant differences with the 7:00 sample and # indicates a significant differences with the preceding sample (P < 0.05). The distribution of data is shown as box-and whisker plots, based on the average, median, quartiles, and extreme values. The box represents the inter-quartile range, which contains the 50% of values. The line across the box indicates the median and the ◊ represents the mean. The "whiskers" extend from the box to the highest and lowest values. Because the data sets were small, no outliers were removed.*

2.3.6.2 The effect of eating a meal on salivary IgA

There was some evidence of a post-prandial effect. Post-prandial s-IgA concentration (see Appendix B), and s-IgA:osmolality ratio was lower after all meals but there was no change in s-IgA secretion rate after any of the meals (Figure 6). S-IgA:osmolality ratio in samples taken at 9:00, 14:00 and 20:30 respectively was either no different or higher than in the pre-prandial samples. Therefore, post-prandial s-IgA:osmolality ratio declines were never sustained for more than 2 hours.

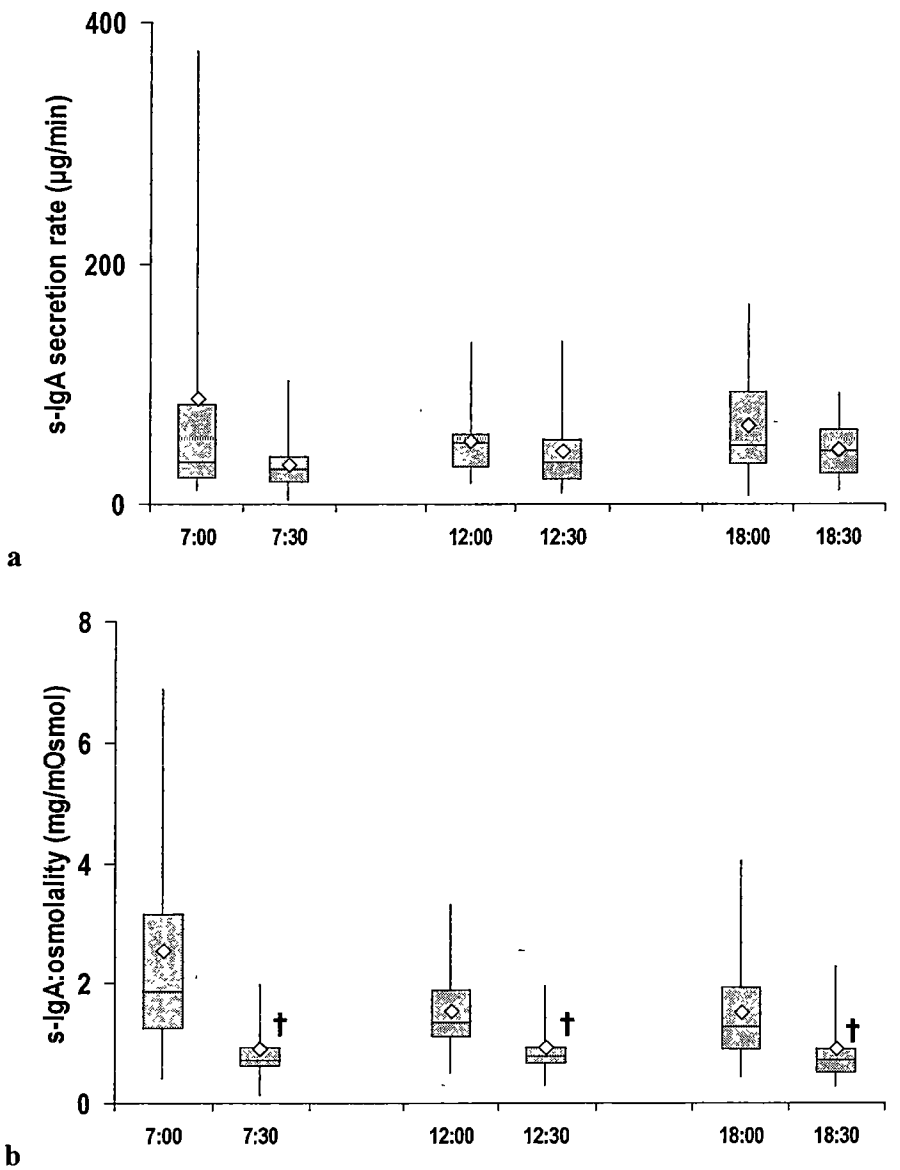


Figure 6: *Eating a meal resulted in a significant reduction of s-IgA:osmolality ratio (b) after all meals, but not s-IgA secretion rate (a): † indicates a significant decrease from pre-prandial samples ($P < 0.05$). The presentation of data is similar to Figure 5.*

2.4 Discussion

2.4.1 Assay performance

Instead of the more traditional ELISA method, a commercial Dade Behring ProSpec nephelometer and a particle enhanced nephelometric immunoassay (PENIA) were used to measure IgA and albumin concentration in saliva. As this equipment is generally used to measure IgA in serum and in CSF and albumin in urine, the available assays had to be optimised.

To fall within the measurable range of the available assays, saliva samples had to be diluted with diluent. The assay precision for IgA was best at a dilution of 1:50. The intra-assay and inter-assay CV_A of 1.8% and 5.2% were lower or similar to those specified by Dade Behring in the product data sheet for the N Latex IgA kit (3.3-6.6% and 3.5-10.1% respectively) and better than those obtained previously by our laboratory using a Dade Behring BNA II nephelometer (4.5% and 5.7% respectively) (Pacqué, 2001). The precision for the albumin assay was best at sample dilutions of 1:10 and 1:25. The intra-assay and inter-assay coefficients of variation of ~ 3% and 6.5% were higher than those specified by Dade Behring for the N antiserum to human albumin (2.2% and 2.6% respectively). As saliva samples were analysed for IgA and albumin in the same analytical run, a 1:10 bench top dilution, combined with machine dilutions of 1:5 for IgA and 1:1 for albumin were recommended. Although this was expected to produce a valid result for the majority of samples, it was envisaged that some samples would need to be re-analysed at a smaller dilution if initial results were below detectable level.

The assay linearity for IgA was good but showed considerable bias for concentrations in the assay range of 4-103 mg/L. There was less bias for the assay range 4-10.3 mg/L. In saliva samples with a bench top dilution of 1:10, this corresponds with a range in saliva of 40 to 103 mg/L, which adequately covers the reference interval (22 - 145 mg/L) established previously by this laboratory (Pacqué, 2001). The accuracy of the methods was very good with an inter-assay CV_A of 4.9% for IgA and 8.3% for albumin. These are within the confidence intervals proposed by the manufacturer for quality control (assigned value ± 20% for the IgA control or ± 15% for the protein control). Because there was no commercial control available to test the accuracy of IgA and albumin measurement in saliva, data on accuracy were obtained from control material designed for serum measurements.

It has been proposed that an assay has a desirable performance if $CV_A < 0.50\ CV_I$ and that its performance is optimum if $CV_A < 0.25\ CV_I$ (Fraser, 2001)). Based on the data from Table 2 and Table 4, the CV_A:CV_I ratio for the IgA assay at a 1:50 sample dilution was 0.09, which corresponds with an optimum performance. Therefore, a 1:50 sample dilution was used for the reminder of this study.

2.4.2 Salivary IgA stability

The addition of sodium azide as a preservative to saliva samples did not interfere with the IgA or albumin assay. This was expected as Dade Behring uses sodium azide ($< 1\ g{\cdot}L^{-1}$) as a preservative in their reagents. The addition of sodium azide made no difference to the stability of s-IgA at storage temperatures ranging from -4°C to +28°C. Therefore, sodium azide was not added to any saliva samples for the remainder of this study.

Sometimes saliva samples are collected in situations where the ambient temperature is high and no cold storage facilities are immediately available. In such circumstances saliva samples are only useful provided s-IgA remains stable until the samples arrive at the laboratory for analysis or can be frozen. It was found that s-IgA was stable for at least six hours when stored at 28°C or room temperature (21°C) but there was a decline after 24 hours. Changes in albumin over the same period were minimal. This result confirmed previous anecdotal evidence from our laboratory. The observed (temporary) increase in albumin concentration after six hours was possibly due to a methodological error, such as an erroneous dilution prior to analysis. The observed changes under these conditions were smaller or only marginally bigger than the coefficients of variation for the assays.

The large increase in s-IgA concentration in samples stored at 4°C and -4°C in the first two to six days was unexpected especially since they were much larger than the small increases in concentration observed for the samples stored at higher temperatures. This was contrary to Butler et al. (1990) who found that s-IgA activity decreased significantly in the first 24 h when stored at 4°C. The increase in concentration can not be explained by an evaporation of saliva water as the samples were stored in sealed containers. Neither could it be attributed to the expected variation in assay results as the percentage changes were much larger than the assay variations. It is possible that a physical change occurred in the samples caused by the chilling and reheating process. Secretory IgA in saliva exists as a dimer, made up of two IgA molecules linked together by a polypeptide (J chain). The breakdown of these IgA complexes into smaller

subunits (monomers) could have exposed extra binding sites which would have been inaccessible to the IgA antibodies in the original dimeric form of the IgA proteins.

Storage of saliva samples at very low temperatures is advised to prevent proteolysis. Even so, storage at -20°C or -30°C might not be enough. For instance in one study, s-IgA concentrations remained stable for up to 3 months at -30°C, but decreased by more than 10% in a majority of samples after 8 months (Ng et al., 2003). Similarly, s-IgA activity to *Streptococcus mutans* in samples stored at -20°C decreased significantly after one week and was completely lost after 18 months whereas storage at -70°C prevented loss of antibody activity (Butler et al., 1990). In this study s-IgA concentration declined significantly in samples stored at -80°C after ~ 70 weeks, whereas albumin concentration did not change in the same samples. Consequently it is recommended that for the determination of IgA in saliva, samples be analysed within a year of collection

Repeated freeze-thawing did not seem to affect s-IgA and albumin concentration, which was in accordance with a previous observation (Mortimer and Parry, 1988). However, from the third freeze-thaw cycle onwards samples showed a visible physical change. They became "cloudy" and a precipitate formed, which could possibly have been caused by a denaturation of proteins in the sample. It has also been observed by others that albumin concentration can decrease by 10% with each additional thaw (M. Gleeson, personal communication 01/09/02). It is therefore recommended to avoid repeated freeze-thaw cycles.

The speed at which samples are frozen has been shown to affect s-IgA concentration. In samples which were not frozen immediately s-IgA concentrations declined by approximately 30% as compared to samples snap-frozen in liquid nitrogen (Nurkka et al., 2003). However, the advantage of snap freezing samples was lost if not applied immediately after collection of the saliva.

2.4.3 Biological and diurnal variation

The large within-subject (CV_I) and between-subject (CV_G) variability for all s-IgA measures were similar to those reported in the literature. For instance Brathall and Waldenstrom found a 15-fold difference in s-IgA concentration between individuals (Bratthall and Widerström, 1985). These differences are not surprising as s-IgA concentration and secretion rate are affected by a multitude of factors such as nutrition, health, fitness and activity levels, environment, stress etc. This study also supported the observation made by Butler et al. (1990) that daily variations in s-IgA are more

characteristic of the subjects than the time of sampling. Some subjects displayed relatively unchanged s-IgA levels throughout a day (see Figure 3; e.g. subject 14), whereas for others they fluctuated widely (e.g. subject 8). This is reflected by the individual CV_I which for s-IgA concentration on the non-fasting day ranged from 24% to 137% and for s-IgA secretion rate from 26% to 98%. For some subjects the results on both days were very similar (e.g. subject 6), whereas for others they were dissimilar (e.g. subject 10). The most consistent phenomenon was the post-prandial effect with 8 subjects out of 13 showing a reduced s-IgA concentration after all 3 meals (see Appendix B).

Although the times of day with the highest and lowest CV_G were different for the various measures there was a trend. On the fasting day, variability seemed highest in the morning and lower in the afternoon. On the non-fasting day variability seemed lowest around 12:00 and highest late in the evening. The best time of day to collect saliva samples for the measurement of s-IgA therefore seems to be between midday and afternoon. As the highest variability was observed in the morning and late in the evening, sampling at these times should be avoided.

This study failed to provide conclusive evidence that s-IgA is subject to diurnal variation but there was some evidence of a post-prandial effect. Several authors have reported a morning high for s-IgA concentration (Gleeson et al., 1990; Hucklebridge et al., 1998; Liu et al., 2003; Richter et al., 1980). In these studies morning samples were generally taken before breakfast after an overnight fast. It was therefore postulated that fasting for a considerable period and/or eating a meal could affect s-IgA levels. However, the present study found no evidence that fasting for one day had an effect on measures of s-IgA as there were no differences in the s-IgA secretion rate and s-IgA:osmolality ratio between a fasting and a non-fasting day. This suggests that the act of fasting in itself is not a major contributor to changes in s-IgA and that the morning high observed by others is not due to the overnight fasting. Our observations suggest that pre-analytical factors, such as increased viscosity of saliva samples due to cellular debris and mucus, could be a more likely explanation of the increased variability of measured s-IgA and apparent changes in saliva concentrations in early morning samples. Generally, first morning samples were unreliable because they were often not suitable for analysis.

In post-prandial samples taken shortly after each meal, S-IgA concentration and s-IgA:osmolality were significantly lower as compared to the pre-prandial samples. Post-prandial declines were sustained for less than 1.5-2 hours and more research is needed

to determine their exact duration and magnitude. S-IgA secretion rate did not change as a result of eating a meal. The cause of the post-prandial effect has not been precisely identified. Factors could include the complex series of hormonal and metabolic changes, which enable digestion of food, absorption of nutrients and the stimulus to produce saliva.

The significant post-prandial fall in s-IgA concentration after breakfast could have been caused by the "waking" effect suggested by Hucklebridge et al. (1998), but only if breakfast was taken immediately after waking. Unfortunately this was not recorded and to provide further proof for this theory, it would be useful to conduct a study with volunteers observing different sleep-wake patterns (e.g. with subjects to "sleep in" late or being woken up in the middle of the night).

2.4.4 Reference interval

The finding that there was a post-prandial decline of s-IgA supports the suggestion that separate reference intervals should be used for fasting and non-fasting subjects (Gleeson et al., 1990). However, as long as samples are collected at least 1.5 – 2 hours post-prandial, a fasting reference interval should suffice.

Reference intervals for the various s-IgA measures were calculated based on samples taken from 134 subjects at least two hours after a meal (Table 10). Early morning (pre-breakfast) or evening samples were avoided. The lower reference value for s-IgA concentration was above the detection limit of the PENIA assay (0.25 mg/L). The reference interval for s-IgA concentration (15.9 – 414.5 mg/L), is similar to the 25 - 600 mg/L published by Gleeson et al. for subjects after an overnight fast but greater than the 9 - 125 mg/L for "non-fasting" subjects in the same study (Gleeson et al., 1990). Discrepancies in the reference ranges of various studies can possibly be explained by differences in the sample size, methodology (e.g. laboratory methods such as equipment, assay method, antisera, etc) and statistical analysis (removal of outliers).

The large biological variation of s-IgA is the main reason for the large range of these reference intervals. This casts serious doubts on their usefulness and is reflected by their low indices of individuality (II). The II reflects the degree of individuality for a measurement within a population and is commonly used for assessing the usefulness of reference values (Fraser, 1994; Solberg, 1994). In general, the lower the II, the higher the degree of individuality. Reference intervals are most helpful when the index is > 1.4 but this is rarely the case for biological analytes. If the II is below 0.6, a population-based reference interval is considered of low utility as a criterion for detecting a

significant change in serial results for an individual. It introduces a high scope for error when comparing an individual's results with the reference interval and the risk of finding "false" positives or negatives is increased. The II in this study were 0.56 for s-IgA concentration, 0.48 for s-IgA secretion rate, 0.64 for s-IgA:albumin ratio, and 0.60 for s-IgA:osmolality ratio.

Figure 3 illustrates the problems associated with using a population based reference interval for analytes with a small II. For an individual, significant clinical changes could occur, even when serial results are all within the reference interval, and consequently remain undetected. It is also possible that results change from inside to outside reference intervals without clinical significance. Strong individuality implies that a subject-specific reference interval would be preferable over a population-based range to assess changes in serial measurements for that individual. Originally it was planned to undertake a project to establish individual reference intervals for a group of subjects. However it was estimated that a very large number of samples would be needed from each subject to obtain a valid reference range. Such a study would also require the control of the many factors which could possibly affect s-IgA and this was beyond the scope of this project.

The large range of the calculated reference in this study is partly due to the statistical method used. Outliers were removed based on Reed's principle. As is clear from Figure 4, data were highly skewed and the use of a different criterion for the removal of outlying data in the right tail of the distribution could have yielded a more narrow reference interval. Despite the fact that more subjects were used than the 120 recommended by the IFCC, the relatively small number of subjects are possibly a limitation of this study. It is possible that a more representative "healthy" reference interval would have been obtained from a larger cross-section of people.

Among the factors, other than time of day and nutritional status, which could affect the reference interval for any biological analyte, are gender, age and smoking status. This study showed that separate reference intervals for males and females are not necessary. This confirmed previous work done at the DSTO laboratory (Pacqué, 2001), which also found no need for stratification of the s-IgA reference intervals between smokers and non-smokers, or (5-year) age groups.

2.4.5 Uncertainty of measurement

There are many and varied sources of variation that can become embedded in a laboratory result. They fall under the broad headings of pre-analytical variation (CV_P),

analytical variation (CV_A), and biological variation (CV_I). Together they contribute to a degree of uncertainty about the validity of any test result.

Pre-analytical variation occurs externally to the laboratory and is mostly outside of its control. Pre-analytical variation in this study was minimised by standardising sample collection, handling and storage methods. Whenever possible, saliva samples were collected in the presence of the investigator. For those collected otherwise, subjects were given detailed and specific instructions.

Sources that contribute to analytical variation may include calibrators, reference materials, equipment, and changes of operator. Further contributors are the precision, bias, interference, linearity, and detection limits of the assay. Analytical variation in this study was limited by standardising all laboratory work and, with a few exceptions, all analyses were carried out by the same operator.

The biological variation of many biological analytes has been quantitated by a number of workers and J. Westgard has compiled an exhaustive list (http://www.westgard.com/biodatabase1.htm). As it is not included in this list, a CV_I for s-IgA concentration was calculated, using the samples from the subjects of the "diurnal variation" experiment. Ignoring post-prandial samples, the CV_I for individuals ranged from17% to 129%, with an average of 55% (see Appendix B). This is of the same order as the average CV_I (42.5%) for a group of 21 subjects who provided an early morning saliva sample three times a week for eight weeks (Pacqué, 2001). Unfortunately, this study was unable to control for the many factors that might affect biological variation, such as diet, stress, hydration, hormonal factors, etc. It has also been suggested that the CV_I depends on the aerobic fitness and activity level of an individual, as in a study comparing groups of elite swimmers, active individuals, and sedentary individuals, the CV_I was 43%, 25% and 24% respectively (Francis et al., 2005).

Despite the differences between individuals, CV_I seems to be the main contributor to the total variation. Assuming pre-analytical variation is minimised, CV_P is irrelevant and total variation can be calculated as $CV_T = (CV_A^2 + CV_I^2)^{1/2}$ (Fraser, 2001). With a CV_A of ~5% and the group mean of 55% for CV_I, the total variation would be ~55%, meaning that the CV_A is practically irrelevant as well.

The considerable total variation has serious implications when it comes to interpreting changes in s-IgA. For serial results to be analytically and biologically different at the 95% confidence limit, they need to differ by $2.77 \times (CV_A^2 + CV_I^2)^{1/2}$ or $2.77 \times CV_T$ (Fraser, 2001, page 73). Thus, using the 55% group mean for CV_T, a change of ~152% would be required for serial s-IgA concentration measurements to be

significantly different. However, the wide range of CV_I between individuals makes it questionable that the use of the group mean for CV_T is the best way to calculate the % change required to "diagnose" a significant change in s-IgA. Instead it seems better to calculate the CV_T for each individual. For instance for the individual subjects in the diurnal variation study, the CV_T for s-IgA concentration ranged from ~18% to ~129%. Consequently, for the individual at the lower end of the range, a significant change would be ~50%, whereas for the individual at the top end of the range it would be ~360%. This reasoning reflects the observation made earlier that individual reference intervals should be used to evaluate changes in serial measurements for an individual.

2.4.6 Recommendations

The measurement of s-IgA by particle enhanced nephelometric immunoassay was found to be a good alternative to other methods as it is automated, rapid, accurate and precise. Based on the results of the work undertaken, following recommendations can be made, which were adhered to for the remainder of this PhD project.

- Care needs to be taken when collecting, storing and analysing saliva samples.
- The time of day at which saliva samples are taken should be accurately recorded and repeat samples should be collected at the same time of day if required for comparison.
- Whenever possible, sample taking should be supervised, especially when accurate timing is needed to calculated s-IgA secretion rate. If this is not possible subjects should be provided with detailed instructions.
- Saliva samples should be taken either before a meal or at least 1.5-2 hours post-prandial. Samples immediately after a meal, early morning (fasting) and late evening should be avoided because of the larger variability at these times, unless required for specific reasons. Early morning samples were often found to be unreliable as they were more likely to contain mucous and contaminants (e.g. cell debris).
- Saliva samples should be analysed as soon as possible after collection. If this is not possible they should be frozen immediately, either by snap-freezing in liquid nitrogen, by placement in dry ice or in a -80 °C freezer. Long-term storage at -80°C can retard the degradation of s-IgA but not indefinitely. Storage in a home freezer (at -4°C or even -20°C) or in normal ice is only useful as a temporary measure.

- In the laboratory, samples can be left at room temperature for a limited time as, even without the addition of a preservative, s-IgA is reasonably stable for a short period.
- Limited freeze-thawing is acceptable but not advisable.

Although s-IgA levels can be expressed in several different ways it is clear from previous work that by doing so a clear pattern often fails to emerge. Depending on the measure used, statistical significance is either obtained or not, and findings are sometimes contradictory. This makes it extremely difficult to reach a definitive conclusion relating to any intervention on s-IgA. To avoid confusion, and to reduce the volume of this work, only s-IgA secretion rate and s-IgA:osmolality ratio are reported in the following experimental chapters.

IMMUNE CHANGES AND UPPER RESPIRATORY TRACT INFECTIONS AFTER AN ULTRA-ENDURANCE RUN

3.1 Introduction

This main purpose of this study was to develop methods for the collection, storage and transport of saliva samples under "field conditions". This study also provided information on the effects of an ultra-endurance running race on mucosal and humoral immunity and assessed if changes in s-IgA and serum IgA correlate with changes in the incidence of upper respiratory tract infection (URTI). It was also assessed if training volume and/or race time was correlated to changes in s-IgA. There is evidence of a relationship between endurance exercise and infections from a large epidemiological study which found that of 2311 participants in a marathon, 12.9% reported an infection during the week after the race in comparison to only 2.2% of similarly experienced runners who had applied to participate in the race but did not do so (for reasons other than sickness) (Nieman et al., 1990a). In a similar study that followed runners during the two weeks after a 56 km ultra-marathon, the overall incidence of URTI was twice as high in participants as compared to age-matched control subjects living in the same household (Peters and Bateman, 1983). This last study also showed a qualitative dose-response relationship between incidence of illness and training volume, a finding which was confirmed in a subsequent study that surveyed a mixed group of 530 runners (Heath et al., 1991). It was therefore hypothesised that the 82 km Cradle Mountain Run would cause a significant, albeit transient immunosuppression.

3.2 Methods

3.2.1 The race

The Cradle Mountain Run (CMR) is an 82 km trail run, traversing Tasmania's Cradle Mountain - Lake Saint Clair National Park. Runners are required to carry a minimum amount of safety equipment and clothing and are responsible for their own nutritional requirements. The weather was ideal for running - overcast in the morning, clearing to a partly cloudy day with minimal rain. The temperature ranged between 5°C and 18°C. High winds were encountered in an early exposed section of the race.

3.2.2 Subjects

Of the 45 entrants in the CMR, thirteen males and four females were recruited into the study. It was assumed that in order to participate in a race of this kind, participants needed to be healthy. Therefore, no exclusion criteria were formulated and no health screening implemented. Subjects were between 27 and 52 years old (mean ± SD; 41.5 ± 8.2), had a body mass index between 20.8 and 23.8 $kg \cdot m^{-2}$ (22.3 ± 1.2) and their race time ranged from 8h 27min to 14h 20 min (11:25 ± 1:32). To estimate their level of physical activity, subjects kept a detailed training diary for a typical week other than the week immediately prior to the race. Activities of daily living were not included. Their average weekly training load was between 4.5 and 15.0h per week (9.5 ± 3.5).

3.2.3 Saliva collection and analysis

Because of the late evening arrival of the subjects at Cradle Mountain, the very early start of the race (6:00am) and the impossibility to obtain samples during the race, saliva samples were limited to late evening and early morning samples. Timed saliva samples were collected in Salivettes the evening before the CMR (18:00 - 20:30), the morning of the race (05:05 - 05:35), as soon as practicable after the race (3 - 30 min after finishing), 2 hours after the race, and the next morning (07:10 - 09:30, before breakfast) (Figure 7). The samples taken the evening before the CMR were collected either before or after the evening meal and the pre-race samples were taken either before or after breakfast. It was not possible to control whether these samples were collected before or after a meal and no record was made of this. Samples were temporarily frozen at -20oC and then stored at -80oC until analysis. The method of collection, storage and analysis of saliva samples was as described in detail in Chapter 2. S-IgA levels are reported as secretion rate and s-IgA:osmolality ratio.

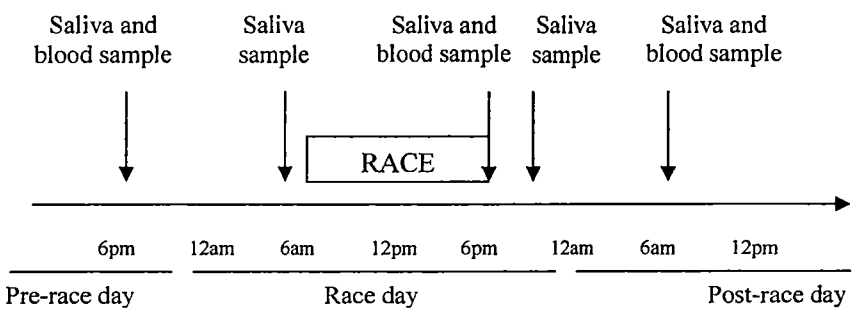


Figure 7: *Timing of saliva and blood samples during the Cradle Mountain Run study.*

3.2.4 Blood collection and analysis

Blood samples were collected from the median cubital vein the evening before the race, as soon as practicable post-race and the next morning (around breakfast time). It was not recorded whether samples were collected before or after a meal. Whole blood was collected in 4 ml Vacuettes (Vacuettes, Greiner Bio-One GmbH, Kremsmuenster, Austria) with anti-coagulant (EDTA). Samples were stored immediately in ice water and processed within 8 hours. A full blood count was performed with a haematology analyser (Coulter MaxM, Beckman Coulter, Fullerton, CA, USA). To obtain serum, blood was collected in 5 ml Vacuettes with a clot activator and serum separator. To allow clot formation, samples were left to stand at room temperature for 20-30 min before being centrifuged. Serum was temporarily frozen at -20°C and then stored at -80°C until analysis. Serum IgA concentration was determined by the same nephelometric immunoassay as described for saliva. All samples were analysed in a single batch. To take into account dehydration, adjustments were made for changes in blood volume (Dill and Costill, 1974).

3.2.5 Upper respiratory tract infections

In the two weeks before and the two weeks after the race each subject recorded the number of days with URTI symptoms, using a self-report questionnaire similar to that used previously (Dick et al., 1987) (Appendix C). Symptoms were defined as common cold symptoms (runny or blocked nose, sore throat, coughing, sneezing, coloured nasal discharge, sinus infection) or influenza symptoms (fever, headache, general aches and pains, fatigue and weakness, chest discomfort, severe cough).

3.2.6 Medication

It is not uncommon for runners in ultra-endurance events to take non-steroid anti-inflammatory drugs (NSAID) to reduce muscle soreness. Subjects were asked to report any such medication taken during the race because of the unknown effect on s-IgA.

3.2.7 Statistical Analysis

Normality of the raw data was tested by the Kolmogorov-Smirnov test. Data for saliva flow rate, serum IgA concentration and lymphocyte counts were normally distributed and descriptive statistics for these data are reported as mean ± SD, range and 95% confidence intervals. Data for s-IgA secretion rate, s-IgA:osmolality ratio, and counts of leukocytes, neutrophils and monocytes were not normally distributed. Analysis of these variables was performed on natural logarithm (ln) transformed data and descriptive

statistics are reported as the geometric mean with back-transformed 95% confidence intervals.

The overall effect of time was evaluated by repeated measures analysis of variance (ANOVA). Differences between individual time-points were assessed by paired t-test. To test correlations between variables, the parametric Pearson's or non-parametric Spearman's correlation coefficients were used. To compare differences between groups of subjects, independent t-tests or the non-parametric Wilcoxon signed-rank test were used. In several instances, lack of sphericity of the data necessitated an adjustment of the degrees of freedom by applying the Huynh-Feldt epsilon (ε) coefficient. For all tests, $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Salivary IgA

Data from two subjects who failed to return all saliva samples were excluded from the analysis of s-IgA. The CMR had a significant effect on s-IgA secretion rate ($F_{4,56} = 10.658$; $P < 0.001$) and s-IgA:osmolality ratio ($F_{2\,3,32.2} = 13.847$; $P < 0.001$). Both measures of immune function were depressed immediately post-race ($P < 0.01$). In addition the S-IgA secretion rate remained depressed until the next morning ($P = 0.009$), while the s-IgA:osmolality ratio had returned to pre race values. Descriptive data are summarised in Table 12 and represented in Figure 8.

Table 12: Changes in s-IgA as a result of the Cradle Mountain Run.

Variable	Evening before	Pre-race	Post-race	2hr recovery	Morning after
s-IgA concentration	206	299	192[§]	82**	235
($mg \cdot L^{-1}$)	(134 - 317)	(207 - 432)	(131 - 281)	(58 - 116)	(162 - 338)
s-IgA secretion rate	159	252	106*	66**	161*
($\mu g \cdot min^{-1}$)	(93 - 274)	(148 - 428)	(58 - 194)	(43 - 100)	(101 - 257)
s-IgA:osmolality ratio	2.2	2.1	0.9*	0.9*	2.4
($mg \cdot mOsmol^{-1}$)	(1.5 - 3.2)	(1.5 - 2.9)	(0.6 - 1.4)	(0.6 - 1.2)	(1.7 - 3.4)
Serum IgA concentration	2142 ± 817	n/a	1640 ± 766*	n/a	892 ± 353**
($mg \cdot L^{-1}$)	(1690 –2595)		(1216 –2065)		(696 – 1087)

Data for serum IgA are mean ± SD (95% confidence interval). All other data are geometric mean (95% confidence interval).

[§] $P < 0.05$, * $P < 0.01$, ** $P < 0.001$, significantly different to pre-race (saliva) or evening before (serum).

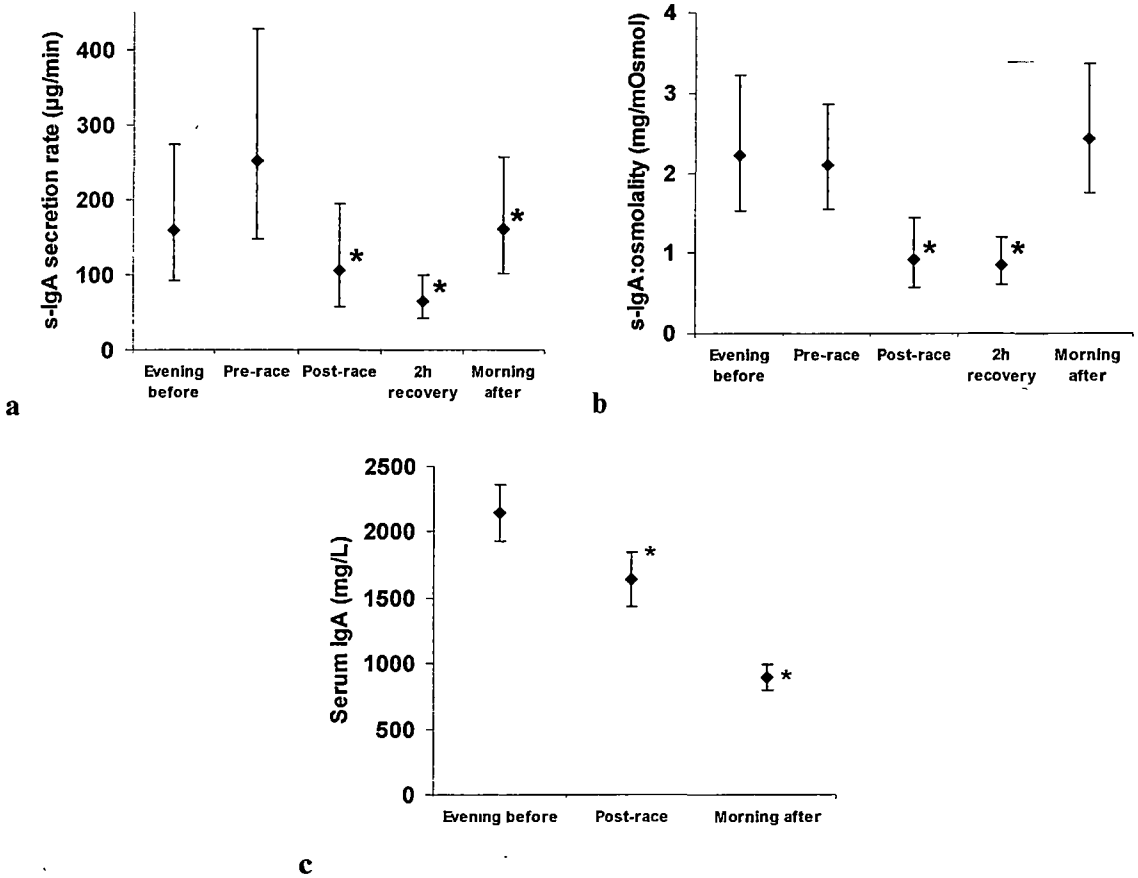


Figure 8: *The effect of the Cradle Mountain Run on various measures of immune function. Post-race s-IgA secretion rate (a) and s-IgA:osmolality ratio (b) were lower than pre-race. Two hours post-race s-IgA secretion rate had further decreased. The next morning, s-IgA:osmolality ratio had recovered, while secretion rate remained depressed (* P < 0.01 significant difference with pre-race). Immediately following the CMR, a significant decline in serum IgA concentration (c) was recorded, which had not returned to normal the next morning (* P < 0.01 change from evening before). Saliva data are represented as the (geometric) mean ± 95% confidence intervals and serum IgA as the mean ± SEM.*

3.3.2 Serum IgA

Data from two subjects who were unable to donate blood after the race were excluded from the analysis. Serum IgA concentration declined significantly as a result of running the race ($F_{2,28} = 26.713$; $P < 0.001$) and declined further during the 24 hour post-race period ($P < 0.001$) (Table 12, Figure 8). To take into account the effects of dehydration, all post-race and recovery samples were corrected for changes in blood volume. However, most subjects finished the race well hydrated and there were no changes in haemoglobin and haematocrit.

There was a significant correlation between s-IgA and serum IgA concentration (Spearman's rho = 0.679, $P = 0.008$) when measured the evening before the CMR. Subsequent changes in serum IgA concentration did not appear to influence s-IgA since the two parameters were not correlated either immediately after the race or the morning after. Neither was there a correlation between their rates of change from pre-race to post race.

3.3.3 Leukocytes

There were significant changes in white cell concentrations across the pre- to post-race period for total leukocytes ($F_{2,28} = 209.5$, $P < 0.001$), neutrophils ($F_{2,28} = 370.2$, $P < 0.001$) and lymphocytes ($F_{2,28} = 10.493$, $P < 0.001$). While the total leukocyte and neutrophil counts increased immediately post-race then partially recovered, the lymphocyte counts showed the opposite pattern, declining immediately post-race then partially recovering (Figure 9, Table 13). Monocyte counts had not changed immediately following the race, but were elevated the next morning.

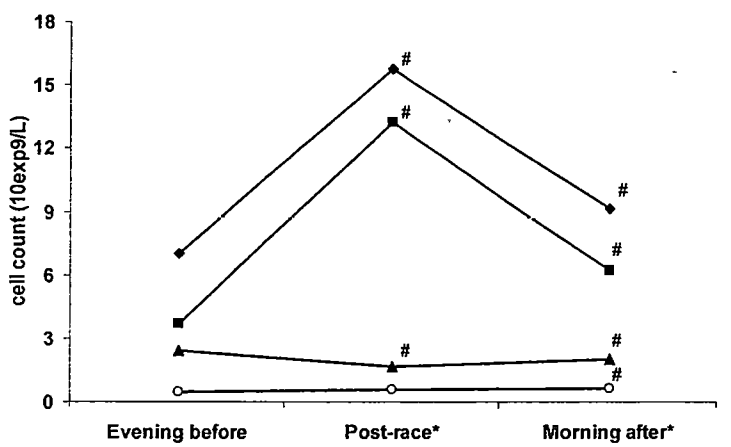


Figure 9: *The effect of the Cradle Mountain Run on leukocyte subsets. Total leucocytes (♦), neutrophils (■), and lymphocytes (▲) but not monocytes (○) had changed significantly by the end off the Cradle Mountain Run. None had recovered the morning after the race. # indicates a significant difference (P < 0.05) with the evening before. *Post-race and morning after data were adjusted for changes in blood plasma volume.*

Table 13: Leukocyte subset counts before and after the CMR

Variable (10^9 L^{-1})	Evening before	Post-race	Morning after
Total leukocytes	7.0 (6.4 - 7.6)	15.8 ** (14.7 - 16.9)	9.2** (8.3 - 10.1)
Neutrophils	3.7 (3.2 - 4.3)	13.2** (11.9 - 14.6)	6.2** (5.5 - 7.1)
Monocytes	0.5 (0.4 - 0.5)	0.5 (0.3 - 0.9)	0.6* (0.5 - 0.8)
Lymphocytes	2.4 ± 0.6 (2.1 - 2.8)	1.6 ± 0.8** (1.2 - 2.1)	2.0 ± 0.7# (1.7 - 2.4)

Data for total leukocytes, neutrophils and monocytes are geometric mean (95% confidence interval). Data for lymphocytes are mean ± SD (95% confidence interval).

* $P < 0.05$, # $P < 0.01$, ** $P < 0.001$, significant change from evening before

3.3.4 Upper respiratory tract infections

During the two weeks before the CMR, 4 subjects recorded a total of 34 days with common cold symptoms. During the two weeks after the CMR, 3 subjects recorded 18 days with similar symptoms. No influenza symptoms were recorded. There was, however, no statistically significant difference in the incidence of common cold symptoms between the two periods ($P = 0.416$). Comparison of subjects with or without URTI symptoms showed no statistically significant differences at any time in s-IgA measures (see Table 14).

Table 14: Differences in s-IgA between subjects with or without URTI symptoms

Variable	URTI two weeks before CMR			URTI two weeks after CMR		
	Pre race	Post race	Morning after	Pre race	Post race	Morning after
s-IgA secretion rate	$P = 0.327$	$P = 0.641$	$P = 0.371$	$P = 0.966$	$P = 0.526$	$P = 0.646$
s-IgA:osmolality	$P = 0.491$	$P = 0.799$	$P = 0.443$	$P = 0.602$	$P = 0.858$	$P = 0.415$

3.3.5 Medication

Nine subjects reported using non-steroid anti-inflammatory drugs (NSAID) during the race, seven said they did not and one subject failed to report on this. Drugs taken were ibuprofen, diclofenac, piroxicam and paracetamol. There was no difference in s-IgA, serum IgA or leukocyte, lymphocyte and neutrophil numbers between those who took NSAID and those who did not (see Table 15). However, the morning after monocyte numbers were lower in the subjects who took NSAID ($P = 0.049$).

Table 15: Differences in serum IgA or leukocyte, lymphocyte and neutrophil numbers between those who took NSAID and those who did not

Variable	Post race			Morning after		
	NSAID	No NSAID	Significance	NSAID	No NSAID	Significance
Serum IgA (mg. L^{-1})	1772	1444	$P = 0.437$	833	980	$P = 0.451$
Leukocytes ($10^9 L^{-1}$)	16.2	15.4	$P = 0.391$	9.1	9.6	$P = 0.401$
Lymphocytes ($10^9 L^{-1}$)	1.8	1.5	$P = 0.455$	2.0	2.1	$P = 0.640$
Neutrophils ($10^9 L^{-1}$)	13.6	13.1	$P = 0.710$	6.4	6.4	$P = 0.995$
Monocytes ($10^9 L^{-1}$)	0.8	0.6	$P = 0.403$	0.6	0.8	$P = 0.049$

3.3.6 Weekly training volume and race time

There was no evidence that weekly training volume correlated with race times (see 3.2.2) or changes in s-IgA. There was, however, a weak but significant negative correlation between race time and post-race s-IgA secretion rate (Spearman's rho = -0.589, P = 0.021). There was no such significant correlation at any other time points. More complete data on the correlations with race time and training volume can be found in Appendix D.

3.4 Discussion

The CMR had a significant effect on mucosal and humoral immunity. The decreases in s-IgA secretion rate and s-IgA:osmolality ratio were almost identical to those reported previously for a 160km ultra-marathon (Nieman et al., 2003) and similar to those reported for a variety of other activities (Gleeson et al., 2000b; Mackinnon et al., 1993a; Steerenberg et al., 1997). In comparison to pre-race values, post-race saliva samples were less voluminous, s-IgA secretion rate declined by 58% and s-IgA:osmolality ratio by 57%. Recovery was slow; in the two hours post-race s-IgA secretion rate declined further and s-IgA-osmolality remained depressed. S-IgA secretion rate failed to return to normal by the next morning. As the morning-after sample was the last one collected, it is impossible to conclude how long it took for full recovery to take place.

The experiments described in Chapter 2 found no conclusive proof of a diurnal variation in s-IgA secretion rate or s-IgA:osmolality ratio. Therefore, there is no reason to believe that a diurnal effect would have contributed to the decrease in these measures during the CMR. The fact that s-IgA secretion rate was still low the morning after the CMR shows the recovery from the effects of the CMR was slow. A significant design fault in this study is the lack of information on meal times in relation to saliva collection times and of particular concern whether the subject was fasting. As this study was carried out

before the study on diurnal variation (see Chapter 2), we were, at the time of this study, not aware of the recommendations that would be made later. It is possible that some of the pre-race samples were taken after breakfast. Unfortunately this was not recorded. As described in Chapter 2, a post-prandial effect could have resulted in a lower s-IgA:osmolality ratio in these samples than if they had been taken before breakfast.

The negative correlation between race time and post-race s-IgA secretion rate indicates that mucosal immune function was more compromised in the slower competitors. This was a short-term effect as there was no such correlation the morning after the race. This finding suggests that for an ultra-endurance activity, exercise duration has an effect on the magnitude of the decrease in s-IgA but does not affect the rate of recovery. The effect of exercise intensity in the present study could not be assessed with validity. It is possible that, regardless of race time, the decline in s-IgA was greater in subjects who ran at a higher intensity. However, exercise intensity was not measured directly (e.g. VO_2 or heart rate). Moreover, race time is not necessarily a fair measure of exercise intensity. To complete a race of this duration, most subjects probably ran at a moderate intensity throughout. While no measurements were taken, it was estimated that the average rate of energy expenditure during the race was 50-75% of the subjects' maximal aerobic capacity. If this assumption is correct, the results of this study suggest that mucosal immunosuppression can occur as a result of moderate intensity exercise.

For all subjects, pre-race serum IgA was within the clinically normal reference range (700 – 4000 $mg \cdot L^{-1}$; Northern Tasmanian Pathology Service). This agrees with a previous finding in 20 marathon runners (Green et al., 1981). Others, however, have found resting serum IgA in athletes after long-term training at an intensive level to be lower than those of control subjects or clinically normal ranges (Gleeson et al., 1995). The acute response of serum IgA to ultra-endurance running has not been extensively studied. Poortmans and Haralambie (1979) found no changes in serum IgA concentration immediately after a 100 km race, which is in contrast with the present study. A 23.4% decrease of serum IgA concentration immediately after the CMR was recorded, with a further decrease to less than half the resting concentration the next morning. Although these changes were highly significant, these depressed values remained within the quoted normal reference range for all but five participants. It is unlikely that such a decline in serum IgA had any adverse health effect, particularly since the incidence of URTI during the two week post-race period did not change, and did not correlate with either pre- or post-race serum IgA concentrations. Furthermore, recent studies suggest that there is maintenance of appropriate antibody response to antigenic challenge despite low serum immunoglobulin

levels in athletes (Mackinnon, 1999a). The clinical significance of the findings of the present study is therefore unclear.

Although salivary and serum IgA concentrations (data not reported) were correlated the evening before the race, s-IgA had partly recovered the morning after the race, whereas there was a continued decrease in serum IgA concentration. This suggests that the measurement of s-IgA can be used as a substitute for serum IgA determinations only when subjects are at rest and have fully recovered from exercise. This proposition needs further investigation.

The resting circulating leucocytes count was normal when compared with clinical norms (4-11 x 10^9 L^{-1}) and with non-athletes (Nieman et al., 1995). However, for twelve subjects the resting leukocyte count was in the lower half of the clinical range. It is possible that their training regime prior to the race caused leukocyte suppression as long periods of intense training have previously been associated with a low resting leukocyte count in distance runners (Lehmann et al., 1997).

The magnitude and time course of changes in leukocyte count as a result of exercise is complex. In general, the increase is higher for more intense and longer exercise, changes can continue even after the end of exercise, and cell counts may remain elevated for several hours after prolonged exercise (Shek et al., 1995). In this study, the magnitude of post race leukocytosis and lymphopenia were consistent with the results of the few previous studies of ultra-endurance runners. Participants in the 90km South African Comrades marathon (Peters et al., 2001) and in a 100km ultra marathon (Gabriel et al., 1994) experienced immediate post-race leukocytosis, lymphopenia and neutrophilia, whereas those in the 160km Western States Endurance Run showed increased counts of leukocytes, neutrophils and monocytes but no changes in lymphocytes (Nieman et al., 2003).

Recovery to resting values was slow: the morning after the CMR, total leukocyte, neutrophil and monocyte counts were still higher than resting values, and lymphocyte count remained low. No further samples were obtained. Only two of the other studies on ultra-endurance runners monitored their subjects for an extended period of time. In the South African study, all blood counts had returned to resting values within 24 hours (Peters et al., 2001). Gabriel et al. (1994) found a 300% increase in leukocyte count, which remained at that level for three hours post exercise. No further samples were obtained

Since blood samples were taken at three time points only, it was not possible to establish the time course of the changes during and after the CMR. It is possible that leukocyte counts in the circulation peaked during the race at levels higher than the post-

race values. During the days following the CMR there could also have been a further decline in leukocyte counts below resting counts. Many of the runners experienced severe delayed onset muscle soreness (DOMS) during that period (personal communications) and exercising for several hours may cause a prolonged suppression of leukocyte counts because of a migration from the circulation, possibly to damaged muscle tissue (Galun et al., 1987). Full recovery to pre-race levels could possibly have taken several days.

Leukocytosis and a change in the subset distribution of leukocytes in the circulation after exercise have been attributed to an endocrine response. A release of hormones, such as catecholamines, corticosteroids, and some cytokines such as IL-1, cause the release of leukocytes from the spleen and changes in the adhesion to the vascular endothelium (Mackinnon, 1999a). It is also possible that cells are simply swept out of the peripheral vessels when blood flow in these vessels increases.

There was no increase in the incidence of URTI following the CMR. This is in contrast with previous findings. Increased symptoms of URTI have been reported in up to 68% of subjects in the two weeks after marathon and ultra marathon races (Nieman et al., 2003; Peters and Bateman, 1983; Peters et al., 1993). This discrepancy could be explained in two ways. First, it should be noted that this study, like some others, relied on "self-report" questionnaires, with possible under- or over-reporting of symptoms or errors in diagnosis. For example URTI symptoms may be confused with allergic reactions (e.g. hay fever). Second, the lower incidence of post-race URTI in this study than in others may possibly be explained by conditions at the finish and the movements of competitors after the race. The window of opportunity for infection post-race as a result of mucosal immunosuppression is limited (Pedersen et al., 1996); there were few participants or spectators, the race was in an isolated environment, and most runners left the finish area soon after completing the race - so their potential exposure to airborne pathogens during the window of opportunity was low. This would be different from many other race situations where participants were more likely to congregate in numbers after the race, with a greater likelihood that someone with an URTI would infect a large number of fellow competitors. Consequently, the low rate of infection could have been due to lack of pathogens rather than any immune effect.

This study did not confirm the finding from some of the studies referred to earlier that the incidence of URTI after ultra-endurance runs increases with "pre-race" training distance (training volume) and race pace. Of the three subjects who presented symptoms of URTI in the two weeks after the CMR, one had the lowest and another had the second highest training volume. Among them were also the third fastest and third slowest

finishers. Because of the low number of subjects in this study, the significance of these findings is limited.

It is not uncommon for runners in ultra-endurance events to take NSAID to reduce muscle soreness. Because it wasn't ruled out that these drugs could have an attenuating effect on (humoral) immunosuppression, subjects were asked to report NSAID taken during the CMR. Although it was observed that monocyte numbers were significantly lower (30%) after the CMR in the group of NSAID takers, this result has to be treated with some caution. A wide range of drugs was taken, not all of which have the same anti-inflammatory effects. Because of the present focus on drugs in sport, it is also quite possible that some subject did not admit to taking NSAID. A better-controlled study is therefore needed before any significance can be attached to this finding.

A study of the relationship between s-IgA and URTI requires a large number of subjects because of the large biological variation of s-IgA. Therefore the number of subjects in this study was too small to look seriously at the issue of the incidence of URTI and the statistical power for the reported measurements is low. However, the primary purpose of this study, which was to develop protocols for the collection, transport and storage of saliva sample, was achieved.

HYPERTHERMIA AND THE MUCOSAL IMMUNE RESPONSE TO LOAD CARRIAGE

4.1 Introduction

To be able to march long distances, whilst carrying heavy equipment is a fundamental aspect of soldiering. Heavy loads are common. Soldiers have been observed carrying loads of up to 81 kg on long-range patrols in the Pilbara (Lau et al., 1999) and Armour Corp soldiers are required to carry loads of 61 kg over variable terrain for up to 5 km (personal communication, M. Paterson, DSTO Melbourne). Heavy load carriage has been shown to lead to an increased incidence of musculoskeletal injuries and increased metabolic cost (Knapik et al., 2004; Patton et al., 1991). Very high levels of physiological and perceived strain have been reported by soldiers carrying modest loads in mildly heat-stressful conditions. In a study by Cotter et al. (2000), only 18 of 31 infantry soldiers who attempted to march 20 km with 35 kg total load finished and only half of these were within the 4 hours time limit. Forty eight of 51 soldiers were able to march 5 km in 55 minutes with a 20 kg load, which is considered relatively light. In both cases heat strain was generally high to severe even though the environmental heat load was not considered high for the location (Townsville, Queensland; average temperature of 27.5°C).

Soldiers often carry loads in more extreme climatic conditions of heat and humidity (e.g. in the tropics). As discussed in section 1.7.4, this could contribute considerably to the physiological stress of exercise. Also to be considered is the problem of heat elimination, when full body cover, such as a Disruptive Pattern Combat Uniform (DPCU), is worn (Shephard, 1998). When fully clothed, the efficiency of evaporative cooling at the skin surface tends to decrease as the sweat accumulates in the clothing, saturates it and has to evaporate from the clothing surface. Once this point has been reached, further sweat secretion has little or no additional cooling effect (Aoyagi et al., 1997). Some of the sweat secreted during load carriage will accumulate under the clothing because of the presence of a backpack. As this surface area is lost to the cooling process, the discomfort and heat stress associated with exercise increases and body temperature will increase. Not surprisingly, heavy training schedules, the carrying of 30-kg packs and the use of protective equipment, including clothing, has lead to many incidences of heat illness among military trainees (Shephard and Shek, 1999b).

The first purpose of this study was to determine whether soldiers marching with heavy loads would experience decreased mucosal immunity, increased physiological and thermal strain, whether any changes would persist after completion of the exercise and whether mucosal immunity is related to changes in thermal strain or perceived thermal strain. The effects of exercise on mucosal immune function were reviewed in section 1.7.

As load carriage is often performed in extreme heat and humidity, the second purpose of this study was to determine whether environmental conditions affect the changes in these variables. The effects of exercise in heat and humidity and immune responses to physical activity in a hot and humid environment have been reviewed in section 1.7.4. It has been proposed that part of the immunosuppressive response to exercise may be due to the elevation of body core temperature associated with exercise (Brenner et al., 1995). It was therefore anticipated that a combination of exercise and environmental stress could have an additive immunosuppressive effect and that the greater hyperthermia, as a result of increases in temperature and humidity, would induce greater immunosuppression.

The third purpose of the present study was to investigate whether the baseline physiological variables of age, body mass, body composition or fitness (VO_{2max}) are related to the changes in physiological strain, perceived thermal strain and mucosal immunity.

It was postulated that heavy load carriage in soldiers would result in temporarily decreased s-IgA secretion rates; that this immunosuppressive effect would be greater in the more stressful environmental conditions; and that the immunosuppressive effect would be greater in the unfit subjects and those with a higher body mass index.

The effect of heavy physical work such as load carriage on soldiers' immune function is of particular interest to the Australian Defence Force (ADF) because it could have a direct effect on the health of their personnel and, therefore, their performance in the field. An infection resulting from immunosuppression in soldiers is likely to reduce performance and operational readiness, just as URTI in athletes may have a negative effect on their ability to compete optimally (Pyne, 1999). The information from this study will give the ADF an insight into the immunological response to a typical field activity and will provide information pertaining to the degree of adjustments in load mass and/or marching speed necessary to maintain operational effectiveness. This study also contributes further to the understanding of s-IgA as a potential biomarker to assess

mucosal immune function or as a predictor of excessive physiological strain and diminished performance, not only in soldiers but also in the general population.

4.2 Methods

This project was conducted in the Human Performance Laboratory at James Cook University, Townsville. Additional approval for this project was obtained from the James Cook University Human Research Ethics Committee.

4.2.1 Subjects

Seventeen apparently healthy male infantry soldiers from the 1st Royal Australian Regiment (1 RAR), stationed in Townsville, Queensland, participated in this study. They were informed of the purpose, risks and benefits of their participation in the project and their decision to participate was entirely voluntary.

4.2.2 Demographics, anthropometrics and fitness

Subjects attended the laboratory for a 1-hr period in the morning in a rested state (i.e. no physical training or physical work was done prior to the assessment). They were familiarised with the experimental equipment and provided with a detailed explanation of the procedures to be used during the three subsequent trials.

Anthropometric measures were obtained according to the guidelines of the International Society for the Advancement of Kinanthropometry (Norton and Olds, 1996): height, body mass (BM), and nine skinfolds (triceps, sub-scapular, biceps, iliac crest, supraspinale, abdominal, front thigh, medial calf and mid-axilla) (Table 16). Body mass index (BMI), % body fat ($BF_{\%}$), body density (BD) were calculated using the LifeSize software (Olds and Norton, 2000). The mean BMI was in the "healthy weight" range as defined by the Australian National Heart Foundation (http://www.heartfoundation.com.au/, accessed 29/04/05). None of the subjects were "underweight" (BMI < 18.5) and 7 were "overweight" (BMI > 25). However, several of these were quite muscular and no-one had a $BF_{\%}$ greater than 20%.

Cardio-respiratory fitness, taken as the maximum rate of oxygen consumption (VO_{2max}), was assessed by an incremental exercise test on a motorised treadmill (Trackmaster, JAS Fitness System, Carrollton, TX, USA, or Quinton Q65, Quinton Instrument Company, Bothell, WA, USA) using open-circuit spirometry. After a 5-min warm-up, the test was initiated with a 2-min run at 11-14 km/h with subsequent increases of 2% incline every 2 min until voluntary exhaustion. Expired gas samples

were measured continuously using a Cortex Metamax I gas analyser (Cortex Biophysik GmbH, Leipzig, Germany). Mixing chamber derived gas exchange data were averaged over 10-sec time intervals. Calibration was performed prior to each test according to the manufacturer's instructions. Heart rate was measured every 5 sec using a telemetric system (Polar Accurex, Polar Electro Oy, Kempele, Finland). VO_{2max} was defined as the highest 30-sec average recorded during the test. VO_{2max} was "average" to "excellent" for males 19 to 25 years old (Shvartz and Reibold, 1990).

Table 16: Demographic, anthropometrical and fitness characteristics of the subjects

Variable	Mean ± *SD*	Range
Age (years)	21.4 ± 2.0	19 – 25
Body mass (kg)	78.8 ± 9.0	64.7 – 98.8
Height (m)	0.178 ± 0.006	0.164 – 0.189
BMI ($kg \cdot m^{-2}$)	24.7 ± 2.1	20.0 – 28.4
Skinfolds (sum of 9) (mm)	98.6 ± 30.3	53.5 – 151.2
% Body fat ($BF_{\%}$)	13.8 ± 3.9	7.6 – 19.4
Body density (BD)	1.067 ± 0.009	1.055 – 1.082
VO_{2max} ($mL \cdot min^{-1} \cdot kg^{-1}$)	53.7 ± 5.0	44.1 – 63.6
HR_{max} (bpm)	192 ± 8	175 - 203

4.2.3 Exercise tests

Subjects attended the laboratory on three occasions at the same time of day at least three days apart. In the 24-hr period prior to every trial they refrained from strenuous physical activity, ate a diet high in carbohydrates and drank at least 1 litre of water before going to bed the night before. In the 12-hr period prior to the trial they abstained from nicotine, alcohol and caffeine ingestion.

The subjects were tested using the ADF's Combat Fitness Assessment (CFA) protocol which requires soldiers to march a set distance at a prescribed pace with a requisite set of equipment. Three subjects were tested simultaneously on three motorised treadmills positioned in a custom built climate controlled chamber. On each occasion, subjects completed a two hour marching trial at a fixed speed of 4.2 $km \cdot h^{-1}$ on a level treadmill.

Subjects carried 50 kg (± 0.05 kg) of equipment, comprised of DPCU, combat boots (~3.5 kg), weapon (~3.5 kg), webbing (~12 kg), loaded pack (~31 kg), and several pieces of body-function monitoring equipment which was not uncomfortable and did

not interfere with the marching activities (< 1 kg). Unloaded walking at a speed of 4.2 $km \cdot h^{-1}$ is a moderate intensity activity, equating to 3-3.3 METS (Ainsworth et al., 1993; Ainsworth et al., 2000).

Trials were terminated if one of the following criteria was attained: completed 2 hr of walking; heart rate exceeded 90% of the subject's maximum heart rate for 3 minutes; body core temperature reached the safe maximum level (39.5°C); the subject indicated their desire not to continue (e.g. due to discomfort or fatigue), felt nauseous or became disoriented; or at the discretion of the investigators due to concern for the subject's welfare.

4.2.4 Environmental conditions

The three marches were done in a random order in three different environments: temperate (T), hot-dry (HD) and hot-wet (HW). These conditions are typical of those encountered in areas of northern Australia where combat-ready troops operate (Table 17).

Table 17: Environmental conditions during the temperate, hot & dry, and hot & wet marches

Temperature (°C)	Temperate (T)	Hot & Dry (HD)	Hot & Wet (HW)
DB: Dry bulb (ambient) temperature	19.6 ± 0.9 (17.6 – 23.7)	28.5 ± 1.3 (25.1 – 31.4)	30.2 ± 1.2 (27.3 – 33.6)
GB: Globe temperature	20.9 ± 0.9 (19.4 – 23.7)	29.6 ± 0.9 (27.3 -31.9)	31.0 ± 1.2 (28.3 – 33.9)
WB: Wet bulb temperature	14.4 ± 0.6 (13.0 – 16.2)	20.4 ± 1.0 (17.7 – 23.5)	25.9 ± 1.3 (22.5 – 30.3)
WBGT*: Wet Bulb Globe Temperature	16.2 ± 0.7 (14.8 – 18.4)	23.0 ± 0.9 (20.6 – 25.8)	27.4 ± 1.2 (24.2 – 33.3)

* WBGT = 0.1DB + 0.2GT + 0.7 WB. All data are mean ± *SD* (range)

Environmental conditions were monitored by a heat stress monitor (MS3700, Metrosonics, Oconomowoc, WI, USA), composed of three thermometers: a wet bulb for humidity determination, a globe (black ball) measuring radiation and a thermometer measuring the temperature. It allows the calculation of the Wet Bulb Globe Temperature (WBGT) as a measure of humidity, temperature and radiation. Air-movement, matched to walking speed, was generated by electric fans (Pedestal Air Circulator 810mm diameter blade, FanQuip, NSW, Australia), and heat radiation,

provided by infra red lamps, was held constant at 500 Watts/m^2 by a solar radiation sensor (Pyranometer SRI,-Kipp & Zonen, Netherlands).

4.2.5 Saliva collection and analysis

Subjects provided a 2-minute timed saliva sample (baseline) during the preliminary visit, at the start and end of each trial, and 45 min after each trial. Samples were collected, stored and analysed according to the procedures recommended in Chapter 2. All sampling was supervised by the investigators. At the end of the project all frozen samples were transported from Townsville to Scottsdale (Tasmania) packed in dry ice. Samples for each environment were analysed in one batch. S-IgA is expressed as secretion rate and ratio to osmolality.

4.2.6 Fluid loss assessment

Subjects began each trial in a euhydrated state. Nude body mass (BM) was obtained at the start and end of each trial. Hydration status was assessed at 30-min intervals by determination of changes in BM. Subjects stoped walking for approximately 1 min when BM was assessed. They were re-hydrated during the trial at 15 min intervals to match fluid losses. Considering some sweat was absorbed in the clothing and equipment, not all fluid lost was replaced during the trial. Subsequently any residual dehydration evident at the end of the trial was eliminated by replacement of fluid equal to 120% of that lost before leaving the laboratory. Subjects also donated a urine sample before and after each trial, to further estimate dehydration levels from the volume and density of urine.

Measurement of urine osmolality would have provided a more accurate assessment of hydration status. A better measurement of fluid loss would also have been provided by the haematocrit of the subjects. However no blood samples were obtained to achieve this. This was a minor flaw in the design of the study.

4.2.7 Physiological measurements

Energy expenditure (metabolic rate) was estimated by calculating oxygen consumption during marching. Expired air was sampled through a half-face-mask for 5-minute periods at 30 min intervals during the trial and analysed for oxygen, carbon dioxide and flow rate in a Cortex MetaMax, gas analyser.

Subjects were monitored continuously for physiological markers of heat-related strain. Core body temperature (T_{core}) was measured at 1 min intervals using gastro-

intestinal radio pills that were swallowed at least 2 hours before the start of work (Pill sensor: HQInc, USA, Logger: BCTM4, Fitsense, MA, USA). Pills were not recovered after use. Heart rate was measured at 1 min intervals, using a sealed transmitter (Polar Accurex, Polar Electro Oy, Kempele, Finland), strapped to the subjects' chest. After completion of each trial, subjects were monitored until core temperature had returned to within 0.4°C of the resting, pre-trial baseline (36.6-37.5°C).

4.2.8 Psychophysical measurements

Perceptions of effort and thermal strain were recorded at 10 min intervals. Subjects rated their perceived work intensity using Borg's 20-point Rate of Perceived Exertion (RPE) scale (ranging from "no exertion at all" to "maximal exertion") (Borg, 1982). Body temperature was rated on a 13-point thermal sensation scale (ranging from "1 - unbearably cold" to "13 - unbearably hot") and a 5-point thermal discomfort scale (ranging from "1 - comfortable" to "5 - extremely uncomfortable") (Gagge et al., 1967). Because the validity of these assessment instruments relies on the compliance of the subjects and the accuracy of their answers, test subjects were thoroughly briefed on their proper use and it is assumed that they were honest in their evaluation.

4.2.9 Data analysis

Outliers in the saliva data were removed according to Reed's criterion, as previously explained. Normality of the raw data was tested by the Kolmogorov-Smirnov test of normality and by coefficients of skewness and kurtosis. Descriptive statistics for normally distributed data are reported as mean ± SD. Non normally distributed data were transformed by natural logarithm (ln) and analysed parametrically, or alternatively a non-parametric test was used. Descriptive statistics of these data are reported as the geometric mean with back-transformed 95% confidence intervals.

The effect of time and environment was evaluated by repeated measures analysis of variance (ANOVA). Lack of sphericity of data was corrected by the application of the Huynh-Feldt epsilon (ε) coefficient. Post-hoc analysis of the effect of the environmental conditions was performed by multiple comparisons with Least Significant Difference (LSD) adjustment. Differences in variables between sampling times or between corresponding sampling times during the three environments were assessed by paired samples t-test or by Wicoxon signed ranks test. To test correlations between predictor and response variables, the Pearson's correlation coefficient was used. For all tests, $P < 0.05$ was considered statistically significant.

4.3 Results

Of the 17 subjects, 15 did the T march, 16 the HD march, and 15 the HW march. Only twelve subjects completed all three marches. On several occasions subjects stopped prematurely because of extreme discomfort (T - 1 case; HD - 2 cases; HW - 2 cases) or because their heart rate was above their theoretical maximum for 3 minutes (HW - 1 case). Partial data from these subjects have been included where appropriate

4.3.1 Physiological variables

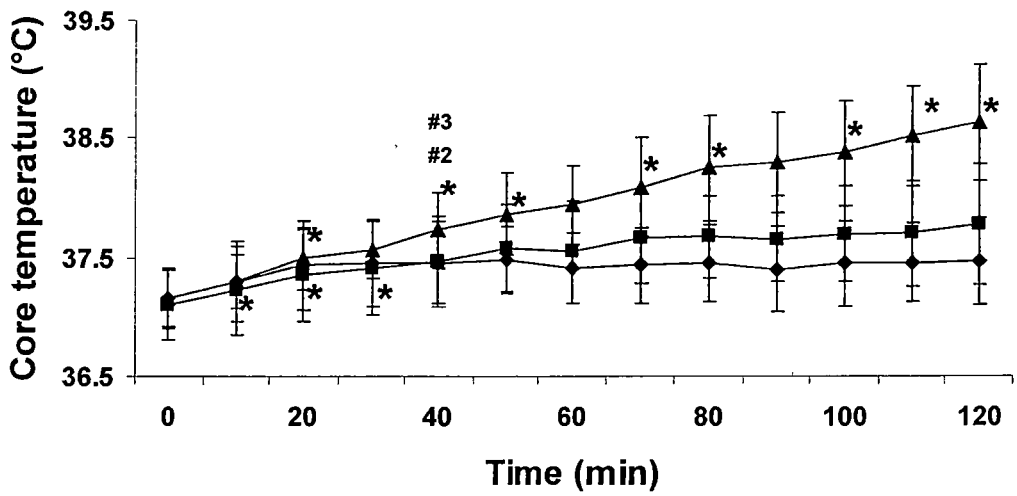
Physiological data are summarised in Table 18 and Figure 10. The change in body mass (BM) corrected for fluid intake and micturition, was significant ($P < 0.001$) in all three environments. All subjects lost BM during each trial with the exception of three subjects who gained some BM (≤ 0.2 kg) during T. The BM loss after HW was greater than after T ($P < 0.05$). There were no changes in the urine specific gravity for any of the three marches. Fluid intake was greater during HW than during T ($P = 0.045$) but there was no difference between T & HD, and between HD & HW.

Table 18: Physiological measurements in three different environments

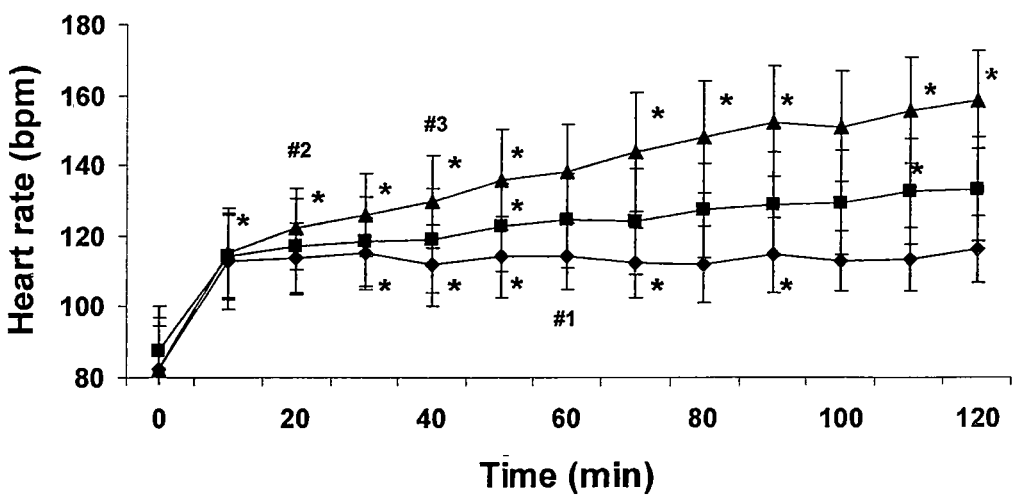
Variable		Temperate (N = 15)	Hot & Dry (N = 16)	Hot & Wet (N = 15)
Change in body mass (BM)	**Gross (kg)**	1.60 ± 0.65	2.14 ± 0.93	2.83 ± 0.51
	Absolute (kg)#	0.36 ± 0.63	0.91 ± 0.73	1.39 ± 0.40
	Relative (% of pre-test body mass)#	0.51 ± 0.94	1.16 ± 0.99	1.74 ± 0.48
Urine specific gravity	**Pre-test**	1.015 ± 0.008	1.018 ± 0.008	1.016 ± 0.006
	Post-test	1.022 ± 0.024	1.018 ± 0.007	1.013 ± 0.007
Fluid intake (mL)		1206 ± 203	1231 ± 373	1437 ± 280
Body core temperature	**Start of march**	37.2 ± 0.3	37.1 ± 0.3	37.2 ± 0.2
(T_{core}) (°C)	**End of march**	37.5 ± 0.4*	37.8 ± 0.5*	38.6 ± 0.5*
Heart rate (HR) (bpm)	**Start of march**	83 ± 14	87 ± 13	82 ± 12
	End of march	116 ± 9*	133 ± 15*	159 ± 14*
	% of HR_{max}	61 ± 6	69 ± 9	84 ± 8
Relative energy expenditure (% of VO_{2max})	**Early in march (10 min)**	36.0 ± 5.9	36.3 ± 6.0	36.2 ± 4.7
	End of march	38.2 ± 5.1*	39.7 ± 5.6*	44.3 ± 5.6*

* Significantly different from "start of march". # Change in body mass corrected for fluid intake and micturition. All data are mean ± SD.

The mean body core temperature (T_{core} - Figure 10a) was identical at the start of all three trials. There was a significantly effect of time ($F_{2\,5,74\,7} = 48.71$; $P < 0.001$) and interaction of time and environment ($F_{5\,0,149\,5} = 16.58$; $P < 0.001$) on T_{core}. During the T and HD trial, T_{core} increased in the first 20min or 30min respectively, and then remained constant, whereas T_{core} gradually increased throughout the HW trial. There was no difference in T_{core} at any time between the T and HD trials but during the HW trial T_{core} was higher than during T and HD after 40min.



a



b

Figure 10: *Changes in core body temperature (a) and heart rate (b) as a result of a 2 hour march in temperate (♦), hot & dry (■) and hot & wet (▲) environments. * indicates a significant ($P < 0.05$) change from the previous measurement. The start of significant differences ($P < 0.05$) between the T and HD, T and HW, or HD and HW conditions, which were sustained for the remainder of the trials, are indicated by #1, #2, and #3.*

The mean heart rate (HR - Figure 10b) was identical at the start of all three trials. HR increased significantly over time ($F_{2.8,105.2} = 194.01$; $P < 0.001$) and the increase in HR was highest in the HW environment ($F_{5.7,210.4} = 23.54$; $P < 0.001$). Apart from a sharp rise in the first 10 minutes, heart rate remained constant during the T trial but there was a significant cardiac drift during the HD trial and even more so during the HW trial. After 120min, heart rates were 61% (T), 69% (HD) and 84% (HW) of the subjects' maximum. Indicative of the increased severity of the HW trial was that several subjects showed distress towards the end and one subject was stopped early because his heart rate exceeded 90% of HR_{max} for over 3 minutes.

Relative exercise intensity, expressed as a percentage of the subjects' VO_{2max}, was identical at the beginning of each trial but had increased by the end in all three ($P < 0.05$). There was no difference between the relative energy expenditure during T and HD but it was higher during HW ($P < 0.01$). At the end of the trials, VO_2 was 38.2% (T), 39.7% (HD) and 44.3% (HW) of the subjects' VO_{2max}.

4.3.2 Psycho-physiological variables

Physiological data are summarised in Table 19. Ten minutes into each of the trials, the RPE ranged from "very, very light" to "somewhat hard". There was a significant effect of time ($F_{2.6,96.6} = 33.24$; $P < 0.001$) and interaction of time and environment ($F_{5.2,193.2} = 3.68$; $P = 0.006$) on RPE. By the end of each trial, RPE had increased ($P < 0.005$), although there was no difference between T ("very light" to "somewhat hard") and HD ("very light" to "hard"). HW ("very light"" to "very, very hard") was perceived to be harder at the end ($P = 0.001$) than the other two.

From the start, thermal sensation during HW ("cool" to "slightly warm") was higher ($P = 0.028$) than during T ("cool" to "neutral") but not during HD ("cool" to "slightly warm"). Thermal sensation increased gradually during all three trials ($P < 0.05$) and by the end was different for all three ($P < 0.005$) (T – "cool" to "warm"; HD – "neutral" to "hot"; HD – "warm" to "extremely hot").

At the start of each trial, thermal comfort was "comfortable" for all subjects. During T it remained unchanged, whereas during HD ("comfortable" to "very uncomfortable") and HW ("comfortable" to "extremely uncomfortable"), it had changed ($P < 0.01$) by the end and was different between all three trials ($P < 0.05$).

Table 19: Psycho-physiological measurements in three different environments

Variable		Temperate (N = 15)	Hot & Dry (N = 16)	Hot & Wet (N = 15)
RPE	**Early in march (10 min)**	9.7 ± 1.9	10.5 ± 1.5	10.5 ± 1.6
	End of march	11.4 ± 1.3*	12.4 ± 2.0*	14.5 ± 2.8*
Thermal sensation	**Start of march**	5.7 ± 0.9	6.2 ± 1.3	6.9 ± 1.1
	End of march	6.9 ± 1.4*	9.0 ± 0.8*	10.3 ± 0.8*
Thermal comfort	**Start of march**	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	End of march	1.1 ± 0.4	1.8 ± 0.9*	2.5 ± 1.3*

* Significantly different from "start of march" or "early in march".

There was little evidence that body composition (body mass, height, BMI, % body fat, skinfolds, and waist:hip ratio) and fitness (VO_{2max}) are good predictors of perceived heat strain and effort (T_{core}, HR, relative VO_2, RPE, thermal sensation, and thermal comfort) as there were few significant correlations between these variables for all environmental conditions. Those that were significant are shown in Table 20. Interactions between predictor variables were not considered in the data analysis.

Table 20: Significant correlations between body composition or fitness and perceived strain or effort at the end of 2h of load carriage

Predictor variable	Response variable at the end of 2 hours of load carriage	Environment	Pearson's r	*P*
Body mass	T_{core}	HD	- 0.645	0.007
	HR	HD	- 0.548	0.042
	Thermal sensation	HW	0.527	0.043
Height	T_{core}	HD	- 0.787	< 0.001
	HR	HD	- 0.564	0.036
	Thermal sensation	T	0.598	0.019
	Thermal sensation	HW	0.638	0.011
% Body fat	Thermal comfort	T	- 0.559	0.030
Skinfolds (sum of 9)	Thermal comfort	T	- 0.518	0.048
VO_{2max}	RPE	T	0.584	0.028

4.3.3 Salivary IgA

Based on samples obtained during the preliminary visit, the group means (± SD; median) of the s-IgA secretion rate was 122 (± 70; 100) $\mu g \cdot min^{-1}$ and s-IgA:osmolality

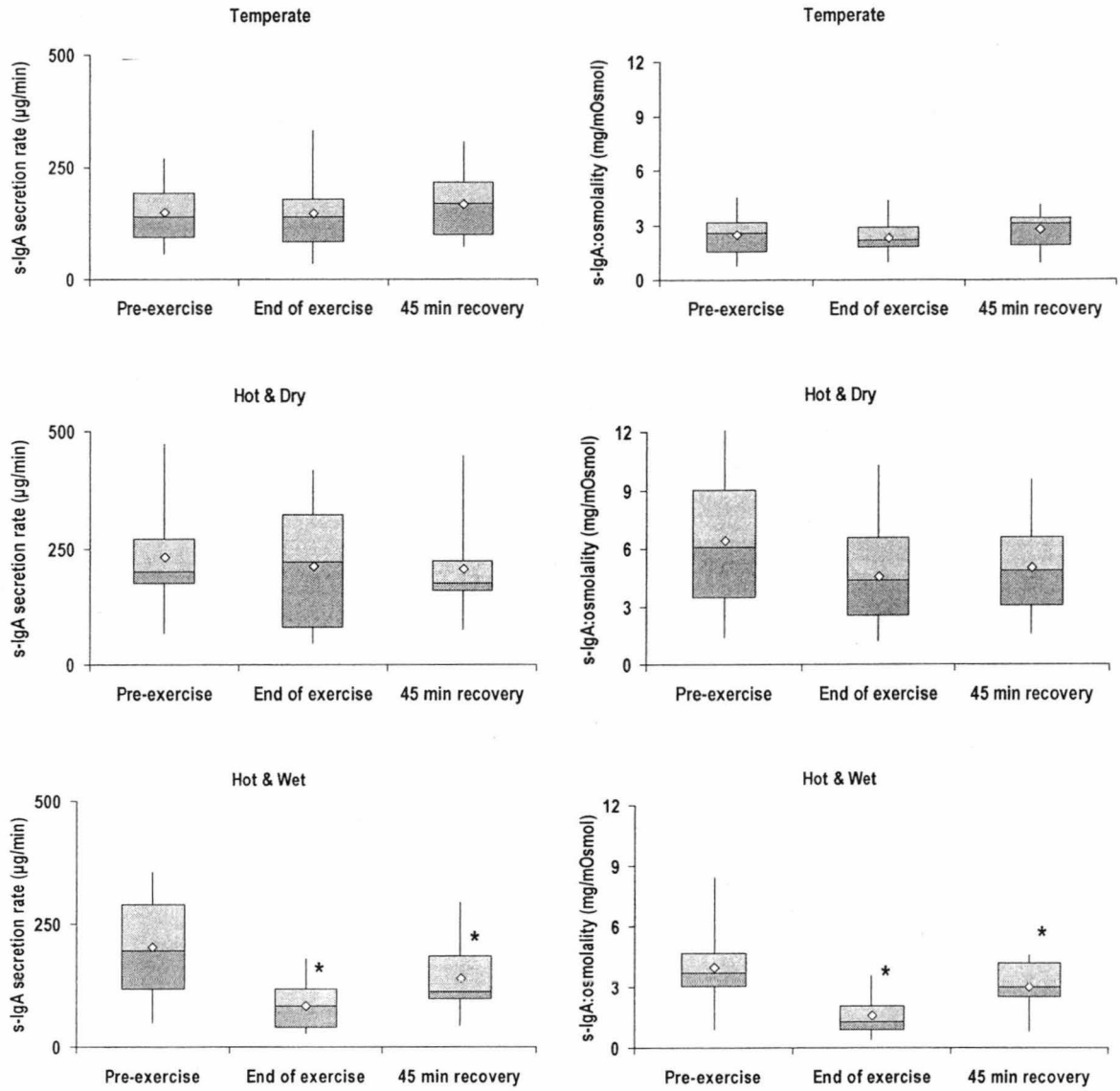
ratio 3.1 (± 1.8; 2.7) mg·mOsmol^{-1}. These baseline values are different to the three pre-test values on the three testing days as listed in Table 21. There is ample evidence in the literature (and this was confirmed by the present study) of the very large variability in s-IgA. The difference in baseline values and pre-test values can be explained by this normal within-subject variation between resting values on the various testing days.

Table 21: Changes in saliva flow rate and s-IgA during a two hour march in three different environments

Variable	Time	Temperate	Hot & Dry	Hot & Wet
Saliva flow rate	Pre-test	0.71 ± 0.30	0.71 ± 0.29	0.71 ± 0.26
(ml·min^{-1})	Post-test	0.69 ± 0.31	0.57 ± 0.24	0.47 ± 0.25*
	45 min recovery	0.68 ± 0.32	0.67 ± 0.29	0.60 ± 0.31
	ANOVA results	$F_{2,28} = 0.122$;	$F_{2,30} = 2.424$;	$F_{2,28} = 6.932$;
		$P = 0.886$	$P = 0.106$	$P = 0.004$
s-IgA secretion	Pre-test	149.9 ± 66.1	205.6 (155.9 – 270.3)	203.5 ± 101.8
rate (µg·min^{-1})	Post-test	146.2 ± 82.6	168.5 (112.0 – 253.4)	83.0 ± 47.2 *
	45 min recovery	166.4 ± 69.8	185.6 (143.8 – 239.3)	139.8 ± 78.0 *
	ANOVA results	$F_{2,26} = 0.506$;	$F_{2,30} = 0.781$;	$F_{16,203} = 11.569$;
		$P = 0.609$	$P = 0.467$	$P = 0.001$
sIgA:osmolality	Pre-test	2.5 ± 1.2	6.4 ± 3.4	4.0 ± 2.2
ratio	Post-test	2.3 ± 1.0	4.6 ± 2.6	1.6 ± 0.9*
(mg·mOsmol^{-1})	45 min recovery	2.8 ± 1.0	5.0 ± 2.3	3.0 ± 1.3*
	ANOVA results	$F_{13,150} = 0.964$;	$F_{2,30} = 3.119$;	$F_{14,168} = 10.840$;
		$P = 0.363$	$P = 0.059$	$P = 0.002$

Data are represented as mean ± SD for normal distributions of data or as geometric mean (95% confidence interval) if data were not normally distributed. * Significant difference with pre-test value ($P < 0.05$).

The changes in s-IgA secretion rate and s-IgA:osmolality ratio during the various trials are summarized in Table 21 and Figure 11 and individual data are presented in Appendix E. Several outliers were removed using Reed's criterion (3 for s-IgA secretion rate, and 4 for s-IgA:osmolality ratio). There were no changes in s-IgA secretion or s-IgA:osmolality ratio as a result of the march in the temperate and hot & dry environments. However, both were lower at the end of the march in hot & wet environment and had not recovered 45min later.



a **b**

Figure 11: *Changes in s-IgA secretion rate (a), and s-IgA:osmolality ratio (b) as a result of a 2 hour march in temperate, hot & dry and hot & wet environments. There were no significant changes in any of the variables as a result of the march in the temperate and hot & dry environments. S-IgA secretion rate and s-IgA:osmolality ratio were lower at the end of the march in hot & wet environments and had not recovered 45min later (* $P < 0.05$, difference with pre-exercise value). The presentation of data is similar to that of Figure 5 in Chapter 2.*

The fitness level (VO_{2max}) of the subjects was not a good predictor of baseline s-IgA or changes in s-IgA measures from pre- to post-test. Likewise, baseline s-IgA was not a good predictor of an individual's response to exercise or perception of work (T_{core},

HR, relative VO_2, RPE, thermal sensation, and thermal comfort). Finally, an individual's response to exercise or perception of work was not a good predictor of post-test s-IgA or changes in s-IgA. Only the few significant correlations between fitness or metabolic response and immune status are shown in Table 22.

Table 22: Significant correlations between fitness levels (VO_{2max}), response to exercise and perception of work (T_{core}, HR, relative VO_2, RPE, thermal sensation, and thermal comfort) at the end of 2h of load carriage and post-test s-IgA or changes in s-IgA

Predictor variable	Response variable	Environment	Pearson's r	*P*
VO_{2max}	Change in s-IgA osmolality	T	0.570	0.042
Relative VO_2	Change in s-IgA secretion rate	HD	- 0.535	0.033
Thermal comfort	Change in s-IgA secretion rate	HD	- 0.507	0.045
Relative VO_2	Change in s-IgA secretion rate	HW	- 0.548	0.043
HR	Post-test IgA:osmolality ratio	HW	- 0.761	0.011

4.4 Discussion

Core body temperature, relative energy expenditure, heart rate, thermal sensation, and perceived exertion all increased during each of the load-carriage marches. Thermal comfort decreased during the HD and HW marches only. The change in T_{core}, relative energy expenditure, and perceived exertion was similar in the temperate and hot dry conditions. There was strong evidence that the increase in all factors was greatest for the hot-wet environmental condition. All of these findings are in accordance with well documented responses to exercise in general and more specifically to exercise in extreme environmental conditions (Gagge et al., 1967; Sutton, 1994).

There was no evidence that marching at 4.2 km·h-1 with a 50 kg load in temperate or hot dry conditions altered the mucosal immune function of fit well-trained male soldiers. However, when these soldiers undertook this activity in a hot-wet environment there was strong evidence for a decline in mucosal immune function (36% for s-IgA concentration, 59% for secretion rate and 60% for s-IgA:osmolality ratio by the end of the march). It appears that the added stress of the hot wet environment resulted in suppression of these measures of mucosal immunity in fit, well trained soldiers. As the study design did not examine the ability of the mucosal immune system to respond to pathological challenge, the results reflect that these parameters were suppressed and not mucosal immunity per se.

The s-IgA secretion rate and s-IgA:osmolality ratio remained low 45min after the completion of the HW march. Several previous studies have shown that s-IgA levels return to resting levels within one hour of cessation of exercise (Cameron and Priddle, 1990; Ljungberg et al., 1997; McDowell et al., 1992a). However, as no further samples were obtained, it was impossible to establish the period needed for full recovery.

This study differs from previous investigations into the effect of environment on mucosal immunity (Housh et al., 1991; Laing et al., 2005; Pacqué, 2001). A previous experiment conducted by the author (Pacqué, 2001), which recruited less fit Army Reserve soldiers in Launceston, Tasmania (mean daily maximum temperature 16.9 °C; mean 9 am relative humidity 65%; - http://www.bom.gov.au/weather/tas/launceston/) provided evidence that mucosal immune function was adversely affected by 2h of load-carriage at 5km·h^{-1} with a 20kg load in both cool-dry (19°C; 51% RH) and hot-wet (28°C; 80% RH) environments. However, there was no difference between the two conditions. In another study, s-IgA secretion rate decreased equally after 2h of stationary cycling at 63% of VO_{2max} in both a hot (30.0°C; 76% RH) and temperate (20.4°C; 60% RH) environment (Laing et al., 2005). Neither was there a significant temperature effect on s-IgA concentration after a 30-minute treadmill run at 80% of VO_{2max} in temperatures of approximately 6, 19, and 34°C (Housh et al., 1991). The usefulness of this last study is limited as only a change in s-IgA concentration was reported. This reflects hydration status and the effect of the exercise on saliva flow rate rather than on any effect on s-IgA production and secretion (Mackinnon, 1999b).

There are several possible reasons why the results of this study differ from previous experiments. The participants in this study were well-trained soldiers (above average aerobic fitness). The fitness level and the "training history" of subjects might affect changes in s-IgA as a result of exercise (Gleeson et al., 2004; Francis et al., 2005). The aerobic fitness of subjects in this study (VO_{2max} : 53.7 ± 5.0 mL·min^{-1}·kg^{-1}) closely matched those of Housh et al (51.3 ± 4.5 mL·min^{-1}·kg^{-1}) but was lower than those of Laing et al (62.2 ± 2.4 mL·min^{-1}·kg^{-1}). While their VO_{2max} was not correlated with baseline s-IgA or changes in any of the s-IgA measures from pre- to post test, it is possible that their high level of fitness and familiarity with this type of exercise influenced the results. Load carriage is an important component of their regular physical training.

It was not recorded how long the subjects had been stationed in Townsville and thus it is not known to what extent they were acclimatised to the local climate which is often hot and humid (mean daily maximum temperature 28.8 °C; mean 9 am relative

humidity 67%; - http://www.bom.gov.au/weather/qld/townsville/). The lack of information on prior acclimation to the hot environment is a serious limitation of this study. It is possible that any immunosuppressive effect in extreme environments is affected by the degree of acclimatisation. This might have influenced the results and could explain why the subjects in this study were less immunosupressed in the T and HD conditions. The degree of acclimatisation possibly also contributed to the variability in results between individuals. A subsequent experiment investigating the effect of fitness and acclimation to heat and humidity seems warranted.

Several methodological limitations of this study may have affected the results. It was recommended that saliva samples collected for comparative purposes should be collected at the same time of day (Chapter 2). Although this was achieved for each individual subject in this study, this was not possible for the group as a whole. The small sample size ($N = 17$), combined with the large biological variation of s-IgA, meant that the power of the statistical tests was low and this might have limited the ability to detect real differences.

The median baseline measurements were in the lower half of the reference intervals established in Chapter 2 (Table 10). Although it is possible that regular physical activity had an effect on both the resting s-IgA and on its response to the load carriage exercise, too little data was collected to substantiate this suggestion. Results of past research are equivocal. Regular exercise had a cumulative suppressant effect on resting s-IgA concentration in elite cross-country skiers (Tomasi et al., 1982) and in Russian Olympic athletes (Levando et al., 1988). Conversely, resting s-IgA was higher in trained rowers than in non-athletes (Nehlsen-Cannarella et al., 2000) and in a group of elite swimmers compared to a moderately exercising control group (Gleeson et al., 1999a). Other authors, however, have found no difference in resting s-IgA between competitive cyclists and age matched untrained control subjects (Mackinnon et al., 1987). It seems that some cumulative changes in mucosal immunity may occur after extended periods of moderate to intense regular exercise. This means that it cannot be excluded that the acute s-IgA changes observed in this study were influenced by the somewhat lower resting s-IgA levels resulting from chronic immunosuppression (Nieman, 1997). However, as only one baseline sample was collected for each subject, and in view of the fact that s-IgA generally displays a large biological variation, this suggestion requires further investigation. The collection of a larger number of "baseline" s-IgA measurements for each subject would be necessary to confirm if it has a predictive effect.

Various mechanisms could possibly explain the observed "hot-wet" effect. By increasing the environmental stress (heat and humidity), the marches were progressively made more demanding physiologically. This was substantiated by the various psycho-physiological measurements and by differences in energy expenditure, heart rate response and core body temperature. The RPE and thermal sensation were generally higher in the HW environment from the start and the ratings of thermal comfort from the halfway point. At the end of the trials, the RPE was higher in the HW environment than in the other two. Thermal sensation and thermal comfort were also rated higher with increasing environmental stress. Several subjects exhibited distress in the final stages of the HW trial: four subjects found the effort "very hard" or above on the RPE scale with one of them also feeling "extremely uncomfortable" and "extremely hot".

The various physiological measures concurred with the psycho-physiological data. The increase in environmental stress resulted in an increasingly higher average energy cost of walking. Average oxygen consumption at the end of the trials was 38.2% (T), 39.7% (HD) and 44.3 % (HW) of VO_{2max}. There was also a significant cardiac drift during the HD trial and even more so during the HW trial with heart rates 61% (T), 69% (HD) and 84% (HW) of the subjects' maximum at the end of the trials.

The more severe environmental conditions during the HW trial resulted in greater hyperthermia. During the T and HD trials, T_{core} remained relatively unchanged after an initial rise, whereas during the HW trial it rose gradually to 38.6 °C (± 0.5 °C), with two subjects exceeding 39 °C by the end of the trial. It has been proposed that moderate hyperthermia (37°C < body temperature < 40°C) enhances the immune system; however, beyond the "therapeutic range" of hyperthermia, immune function becomes compromised (Brenner et al., 1995). The greater decline in s-IgA in the HW environment in this study confirmed that this is to some extent the case for the mucosal immune system.

It is possible that some of the changes in s-IgA were temperature-induced (Brenner et al., 1995) because some of the effects of hyperthermia on the immune system are similar to the changes observed in exercising subjects (Hoffman-Goetz and Pedersen, 1994; Shephard and Shek, 1999b). However, as studies on the effects of exercise on s-IgA rarely recorded changes in T_{core}, there is little evidence to substantiate this claim. The design of the present study made it also impossible to separate the individual effects of the physical activity and hyperthermia and it is impossible to conclude that it was the sole cause.

One other factor that could have contributed was the increased psychological stress as the environmental conditions became more taxing. The subjects' ability to cope psychologically with the load and the heat and humidity was probably important. As psychological stress has been found to result in changes in mucosal immunity, it is likely that there was an additive response to the combined stimuli of hyperthermia, physical activity, and mental stress. This is possibly mediated by changes in the endocrine system, i.e. an increase in the blood concentration of stress hormones (Hoffman-Goetz and Pedersen, 1994; Kappel et al., 1997; Kappel et al., 1991). While a minimum increase of body temperature might be needed to initiate a significant temperature-induced stress hormone response (Hoffman-Goetz and Pedersen, 1994), it is possible that the heat exposure in the HW environment in this study was above the threshold needed or at least magnified the response. More well designed studies are necessary to separate the individual effects of psychological stress, exercise and hyperthermia on s-IgA.

There was no evidence that body composition or fitness are good predictors of perceived heat strain and effort during load carriage in a fit and healthy population. This might not be the case in a population group where there is greater variability in fitness level and body composition measures. It was assumed that the fitter and/or "bigger" subjects would cope better with the load, but not necessarily with the heat and humidity. This was not consistently observed across the three environments. Neither was there any evidence that fitness is a good predictor of resting s-IgA secretion rate or changes in s-IgA secretion rate as a result of load carriage. Finally, there was no evidence that resting s-IgA secretion rate would be useful as predictors of a person's perception of work in any environment. Because of the inconsistent findings, it is possible that the few significant correlations in Table 21 and Table 22 could have occurred by chance rather than being indicators of real associations.

As the interpretation of the data in this chapter is based on group means, this can overlook important information when individual subjects are assessed. As shown in the table with individual results (Appendix E), a considerable within-subject and between-subjects variability in s-IgA responses was observed. For instance, changes from pre-test to post-test s-IgA secretion rate varied between a 93% decline to a 58% increase for the HW environment. Of the 12 subjects who completed all three marches, only a few responded consistently, showing either a decline or an increase in s-IgA levels in all three environments (2 for s-IgA secretion rate; 4 for s-IgA:osmolality). The reason(s) for the considerable variability in s-IgA responses found in this and other studies remain to be

clarified. None of the factors, such as psychological stress, nutrition, and improper hygiene that have been associated with immunosuppression (Nieman, 1997) were controlled for in this study. Individual differences could also be due to different endocrine responses to exercise at various temperatures (Housh et al., 1991). The findings of this study do not support the suggestion that differences in fitness might account for the range of responses (Mackinnon, 1996) as no correlations were found between the aerobic fitness (VO_{2max}) of the subjects and their resting s-IgA or s-IgA changes.

In summary, the present work identified a transient decrease in mucosal immunity and increased stress in response to two hours of load carriage in a hot-wet environment. It is recommended that soldiers involved in military training and military operations in similar conditions are regularly monitored for excessive signs of fatigue and dehydration, which could compromise their physiological and cognitive performance. Although there could be a degree of extra risk of infection associated with the type of immunosuppression observed in this study, it is doubtful that s-IgA is a suitable marker for regular monitoring of mucosal immune function.

SALIVARY IgA, MILITARY TRAINING AND OVERTRAINING SYNDROME

5.1 Introduction

The Army Recruit Training Centre (ARTC) is located at Blamey Barracks, Kapooka, near Wagga Wagga in New South Wales. The primary role of the ARTC is to provide basic training for new recruits for the Regular Army and the Army Reserves of the Australian Defence Force (ADF). The 45-day Army Common Recruit Training (ACRT) course is essentially the same for male and female recruits and involves activities ranging from 40-minute lessons (sit-down classes, weapon handling and firing, first aid, drill, navigation, communication, physical training sessions and field craft) to longer training sessions in the field. Some of the more arduous activities are marches in full combat gear and bayonet drills. Training is conducted continuously over the 45 days with most days commencing at 06:00 h and finishing at 22:00 h. The ACRT culminates in a 3-day field exercise called "Exercise Dusty Warrior" (EX DW - days 39-41) followed by "The Challenge" (day 42), lasting 5½ hours (6 am to 11:30am). During these exercises, the recruits' stamina, determination and ability to work in a team and their ability to put all the field craft skills they have learnt into practice is tested. The ACRT course is both physically and psychologically demanding, leaving recruits with very little time to recover from training and relax. All activities, including personal recreational time, are scheduled rigorously so that the demanding requirements of the course can be met within the 45-day timeframe. Recruits often face a variety of stressful situations. These can be caused by any number of factors such as injury, the very full time table, the limited personal time and privacy, the pressure to perform, a bad relationship with superiors, etc. Not surprisingly, most recruits require long recovery times after physically demanding activities and have difficulty remaining alert during classroom based lessons. Some recruits fail to complete the course because of choice, stress, illness or injury.

Overtraining syndrome (OTS) is a condition often used to describe athletes suffering from prolonged fatigue and impaired athletic performance. It could possibly also occur in response to demanding training or workloads in population groups such as army personnel. As OTS has previously been associated with immunosuppression (Budgett, 1990) and a high rate of URTI in athletes (Fitzgerald, 1991), the first aim of this study was, therefore, to assess if there were changes in mucosal immunity during

the ACRT course and if these were related to an increase in the incidence of URTI. An overview of the relationship between salivary IgA and the susceptibility to URTI can be found in section 1.5 and section 1.8.3 deals with the association of OTS with changes in salivary IgA and susceptibility to URTI. There is also some evidence that s-IgA is suppressed in athletes during prolonged periods of intense exercise training leading to OTS. For instance, among 14 elite swimmers over a 6-month season, s-IgA levels were lower in overtrained swimmers compared with those who did not show signs of overtraining (Mackinnon and Hooper, 1994). As there is possibly an increased risk of URTI among endurance athletes, and a dose–response relationship between training volume and incidence of URTI, it appears that both OTS and URTI could result from a common cause: excessive training with insufficient rest and possibly a lack of variety in training. Because the recruits at the ARTC are engaged in a six-week intensive training program, and as there was anecdotal evidence from the instructors and officers at the ARTC that the demands of the ACRT course cause many recruits to suffer from various manifestations of fatigue, it was postulated that recruits could be susceptible to OTS.

This study was part of a much larger study conducted by the Defence Science and Technology Organisation (DSTO) investigating if army recruits display symptoms of overtraining. The larger study was the subject of a DSTO report (Skiller et al., 2005b) and a journal article in "Military Medicine" (Booth et al., 2006). The purpose of this study was not to investigate if soldiers showed any symptoms of OTS. The question this study aimed to address was whether s-IgA would be a potential alternative marker of overtraining syndrome. An overview of the various markers that have been associated previously with OTS is presented in section 1.8. The second aim of this study was therefore to investigate if there was an association between s-IgA measures (or changes in those measures) and the variables (or changes in those variables) identified by the DSTO as possible indicators of OTS: these include physiological variables (weight, physical performance); fatigue; mood states; and haematological and biochemical variables (leukocytes; neutrophils; lymphocytes; monocytes; neutrophil to lymphocyte ratio; C-reactive protein; TNFα; haemoglobin; ferritin; free testosterone; cortisol; FTCR). To allow this question to be answered, some of the results of the larger DSTO study were reproduced (with permission from the DSTO).

5.2 Methods

5.2.1 Subjects

Of the 58 recruits (51 males, 7 females) who volunteered for the study, 43 (38 males, 5 females) completed the ACRT course and the study. Nine males were cigarette smokers. The gender bias in this study reflects the fact that the proportion of females in the Army is generally less than 20%. Except for the health checklist all tests and activities associated with this study were scheduled into the formal ACRT timetable.

5.2.2 Physiological measurements

5.2.2.1 Anthropometry

Body mass and height were recorded before breakfast on days 1, 33 and 43. Subjects were asked to drink at least 0.5 L of water the previous night to ensure a good hydration status. The methods for recording anthropometric data were based on those of the International Society for the Advancement of Kinanthropometry (Norton and Olds, 1996).

5.2.2.2 Energy expenditure

Total Energy Expenditure (TEE) was estimated by the factorial method based on observations of activities and assigning values for the energy costs of each observed activity (Ainsworth et al., 1993; Ainsworth et al., 2000). Detailed records of recruits' activities were made on days 9, 35 and 38 and during The Challenge and have been described in a related publication (Skiller et al., 2005a). It was not possible to directly observe subjects during EX DW for security reasons; therefore TEE was estimated using information and advice from ARTC staff about the mode, intensity and duration of the exercise. To estimate the mean daily TEE, each day's activities were classified as intense (e.g. physical training lessons, marching to and from lessons, endurance marches, obstacle courses, field-training lessons involving high levels of exertion), moderate (e.g. daily tasks, military activities such as drill, training in living accommodation and other tasks allocated by training instructors), and sedentary (e.g. lectures, eating, resting while waiting for lessons, sleep and similar activities). The TEE for each day was estimated based on the breakdown of time spent on the various activities and by use of subjects' calculated basic metabolic rate (BMR).

5.2.2.3 Physical fitness

Fitness testing was conducted on days 3 and 32 during regular physical training sessions under the supervision of the physical training instructors at ARTC. A 2.4 km run was used as a measure of aerobic capacity and endurance (Burger et al., 1990), maximum number of push-ups as a measure of upper body strength and standing vertical jump as a measure of leg muscle power (Suni et al., 1996).

5.2.3 Psychological and health measurements

5.2.3.1 Fatigue

Fatigue was assessed on days 1, 6, 13, 20, 27 & 34 by the 30-item Multidimensional Fatigue Symptom Inventory (Short Form) (MFSI-SF; Appendix F). This is a validated questionnaire sensitive enough to detect changes in fatigue over the short period of time in the present study (Stein et al., 2004). It contains no reference to medical conditions so it can be used by both healthy and ill individuals. It does not assume the presence of fatigue, so it can be used to gather baseline data before an expected fatigue-inducing event/activity. The subscales can be compared. For example individuals may be free of physical fatigue symptoms but have symptoms of other fatigue dimensions such as emotional fatigue. Subjects rated their experience of each symptom on a 5-point Likert scale as how true they were for them in the past 7 days (i.e. not at all, a little, moderately, quite a bit or extremely). The item scores combine to produce five subscales measuring different dimensions of fatigue: general fatigue, physical fatigue, emotional fatigue, mental fatigue and vigour.

5.2.3.2 Mood

Changes in mood state were monitored on days 1, 6, 13, 20, 27 & 34 by the Profile of Mood States (POMS) questionnaire (Mc Nair et al., 1971). Subjects rated the 65-item list of words relating to how they had been feeling during the past week, according to a 5-point Likert scale. The POMS was scored for each of the factors, tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, confusion-bewilderment, and total mood disturbance.

5.2.3.3 General health

Subjects completed a daily health diary every morning, using a simplified 9-point health checklist, which included symptoms of URTI (Appendix G).

5.2.4 Biochemical measurements

5.2.4.1 Saliva collection and analysis

Although saliva samples were collected for all 58 subjects, only the samples of 30 subjects who provided a complete set of samples were analysed. This was both for financial and statistical reasons. Analysing all samples was beyond our budgetary means and incomplete sets would have complicated statistical calculations. A total of 690 saliva samples were collected from 30 subjects (23 samples each):

- Early morning samples (before breakfast; ~ 6:00 h) on days 1, 2, 13, 27, 32, 38, 39, 40, 41 & 43.
- Late afternoon samples (before dinner; ~ 17:35 h) on days 2, 3, 4, 5, 6, 20, 34, 38, 39, 40, 41, & 43.
- Before and after The Challenge on day 42.

Saliva was temporarily stored in a -80°C freezer and transported to the DSTO laboratory in Scottsdale, Tasmania on dry ice. Otherwise all saliva samples were collected, stored and analysed as described in Chapter 2. S-IgA levels are reported as a secretion rate and as a ratio to osmolality.

To assess the overall effect of the ACRT on s-IgA, 4 time points were considered: week 1, day 20, day 34 and week 6. To this effect, the results of the pre-dinner saliva samples of week 1 (days 2, 3, 4, 5 & 6) and week 6 (days 38, 39, 40, 41 & 43) were averaged for each subject. The reason for deciding to average the data of the first and last week was that there is a large within subject variability in s-IgA. Despite the fact that week 6 included the three samples collected during the strenuous training period and three on other days, the average was deemed to give a better idea of an individual's representative s-IgA status, rather than the results from a single day.

The most strenuous period of the ACRT was from day 39 to day 42 with Exercise Dusty Warrior, followed by The Challenge. Early morning (pre-breakfast) and late afternoon (pre-dinner) saliva samples were compared with samples taken on day 38 (baseline) and day 43 (recovery sample).

5.2.4.2 Blood collection and analysis

Blood samples were collected after an overnight fast (before breakfast) on days 1, 33 and 43 from a superficial antecubital vein into an EDTA tube (10 mL, BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and a plain serum tube, containing a clot activator and a gel separator (9.5 mL, Vacutainer SST Gel & Clot

Activator, Becton Dickinson, Franklin Lakes, NJ, USA). A basic haematological profile (haematocrit, haemoglobin concentration, red and white cell counts) was performed on whole EDTA blood within 10 hr of collection by use of routine methods (Beckman Coulter MAXM automated analyser, Miami, Florida, USA). The ratio of neutrophils to lymphocytes was calculated as a suggested index of the physiological stress on the immune system (Nieman et al., 1999). The serum free testosterone to cortisol ratio (FTCR) was used to determine the onset of overtraining (Chicharro et al., 1998a). Other indicators of overtraining, namely ferritin (Frt), C-reactive protein (CRP) and in vivo inflammatory cytokine (tissue necrosis factor alpha, TNF_{α}) were also measured from the serum sample.

Samples were batch analysed, ensuring that each subject's samples were analysed within the same day. Reagents from the same production batch were used throughout the analysis. Standard commercial methods were used in the analysis of all blood analytes: free testosterone (Coat-A-Count Radioimmuno assay, DPC Cirrus Inc, Los Angeles , California, USA), TNF_{α} (Accucyte competitive enzyme immunoassay, Cytimmune, Maryland, USA), Frt and high-sensitivity CRP (ProSpec auto-analyser, Dade Behring, Marburg, Germany), and cortisol (Vitros Eci, Johnson & Johnson Clinical Diagnostics Inc, Rochester NY,USA).

5.2.5 Statistical analysis

Descriptive statistics are presented as means, standard deviations, and range or 95% confidence interval. Data was initially checked for outliers using Reed's criterion (Fraser, 2001). Normality of the raw data was tested by the Kolmogorov-Smirnov test, the Shapiro-Wilk or the Lilliefors tests. Where needed, normality was achieved by ln or log transformation. Significance was accepted at $P < 0.05$. Associations between variables were assessed by Pearson's correlation coefficient or by multiple linear regression analyses. For some relationships Spearman's rank correlation was used if the complex natures of the relationships precluded the use of parametric regression methods. Comparison of means was achieved by use of the paired t-test with Levene's test used for comparison of variance. Repeated measures analysis of variance (ANOVA) was used for procedures with serial measurements. The results of all within-subject F-tests were based on Huynh-Feldt corrected *P*-values. Two forms of contrasts were used in the comparison of variables across time: (1) comparison of each time against a baseline was used as the primary contrast, and (2) comparison with the previous time

was used to increase an understanding of where changes occurred. Contrasts were only used when there was evidence of a difference across time from the primary test.

To establish the predictive capability of s-IgA measures on changes in other variables, regression analysis was employed using the non-saliva variables as the response variables and the saliva variables as predictors. The predictive role of s-IgA measures was examined both as individual predictors and as collective predictors through the use of multiple linear regressions with stepwise fitting of variables. The response variables were formed as the difference between the baseline response and responses at selected times during the study. Differences in saliva variables were calculated to cover the corresponding time periods.

5.3 Results

5.3.1 Subjects

The mean age of subjects was 22.0 years for males (range 18 - 33) and 29.5 years for females (range 19 - 46). Mean height was 179.5 cm (range 164 – 196) for males and 165.0 cm (range 160 - 171) for females. Mean body mass for males was 78.8 kg (range 60 - 104) and females 64.5 kg (range 57 - 74). The mean BMI was 24.3 $kg.m^{-2}$ (range 20.1 - 29.4) for males and 24.3 $kg.m^{-2}$ (range 21.1 - 28.6) for females. Of the 38 males and 5 females who completed the study, 14 and 2 respectively were overweight (BMI $\geq$ 25) and none were obese (BMI $\geq$ 30).

There was a small but significant body mass loss among the males (mean = -2.5 kg; $P < 0.001$) and a non-significant loss among females (mean = -2.0 kg; $P = 0.095$) who finished the course.

5.3.1.1 Energy expenditure

On any day most recruits were possibly engaging in a greater level of physical activity than would have been normal for them prior to their participation in the ACRT. The recruit's average daily total energy expenditure (TEE) was $16.5MJ.day^{-1}$, which would be considered extremely high for their relatively sedentary civilian peer. During the first four weeks the intensity level was relatively stable, with the TEE 16.5 $MJ.day^{-1}$ and 12.4 $MJ.day^{-1}$ for males and females respectively. In weeks 5 and 6 and at the beginning of week 7 there was an increase in the physical activity level, particularly during EX DW. On the most arduous day of EX DW the energy expenditure was approximately 28 MJ for male recruits (Table 23).

Table 23: The total daily energy expenditure (TEE) of male and female recruits during the 45-day ACRT

	TEE ($MJ \cdot day^{-1}$)			
	Males		Females	
Day Type (number of days)	**Mean**	**(Range)**	**Mean**	**(Range)**
Standard day (33)	16.3	(14.0 – 18.6)	12.1	(11.7 – 12.6)
Day with increased physical activity (8)	18.0	(15.5 – 20.6)	13.4	(12.9 – 14.0)
Day of the Challenge (1)	22.3	(19.2 – 25.5)	16.6	(16.0 – 17.3)
EX DW - Day 1 (1)	19.7	(16.9 – 22.5)	14.6	(14.1 – 15.2)
EX DW - Day 2 (1)	27.6	(23.8 – 31.6)	20.6	(19.8 – 21.4)
EX DW - Day 3 (1)	22.3	(19.2 – 25.5)	16.6	(16.0 – 17.3)
All days combined (45)	**17.2**	**(15.1 – 19.4)**	**12.8**	**(12.5 – 13.2)**

5.3.1.2 Physical fitness

There was an increase in upper body strength ($P < 0.001$, push-up test) and leg muscle power ($P < 0.001$, standing vertical jump) but a small decline in aerobic capacity ($P < 0.001$, 2.4 km run) (Table 24).

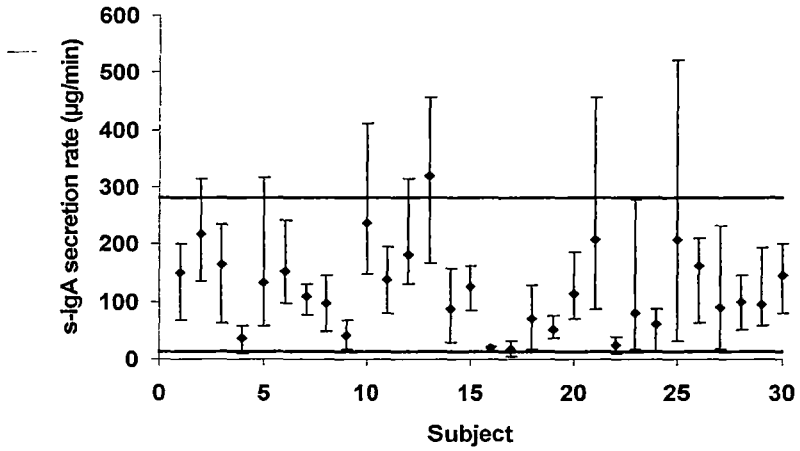
Table 24: Physical fitness of recruits during the ACRT

Variable	Day 3 (N=58)	Day 32 (N = 40)
Upper body strength (number of push ups)	33 ± 12 (11 – 69)	40 ± 11* (16 – 69)
Leg muscle power (standing vertical jump in cm)	67 ± 17 (27 – 100)	72 ± 18* (28 – 100)
Aerobic capacity (time for 2.4 km run in $min.sec^{-1}$)	10:11 ± 1:13 (8:09 – 13:17)	10:32 ± 1:02* (8:44 – 13:28)

Data are mean ± *SD* (range) for males & females combined; * Significant change from day 3

5.3.2 Biological variation of salivary IgA

The pre-dinner saliva samples from days 2 to 6 were used to calculate the between-subject and within-subject variability and the indices of individuality of the s-IgA measures (Table 25). The individual data for all subjects has been included in table format in Appendix H. Figure 12 graphically represents the spread of individual data for each subject and compares them with the reference ranges calculated in Chapter 2 (Table 10). Mean s-IgA secretion rates differed by up to 18-fold between subjects.



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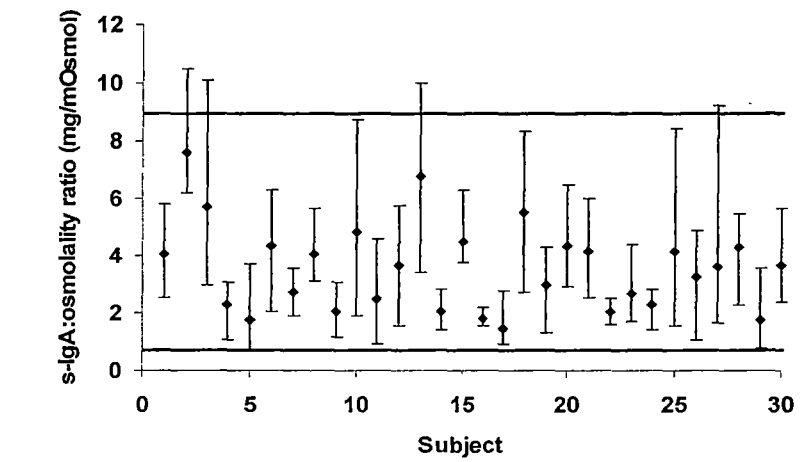

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Figure 12: *Mean (♦) and absolute range of s- IgA secretion rate (a) and s-IgA:osmolality ratio (b) for 30 healthy subjects during the first week of the ACRT course. The horizontal lines represent the reference range calculated in Chapter 2.*

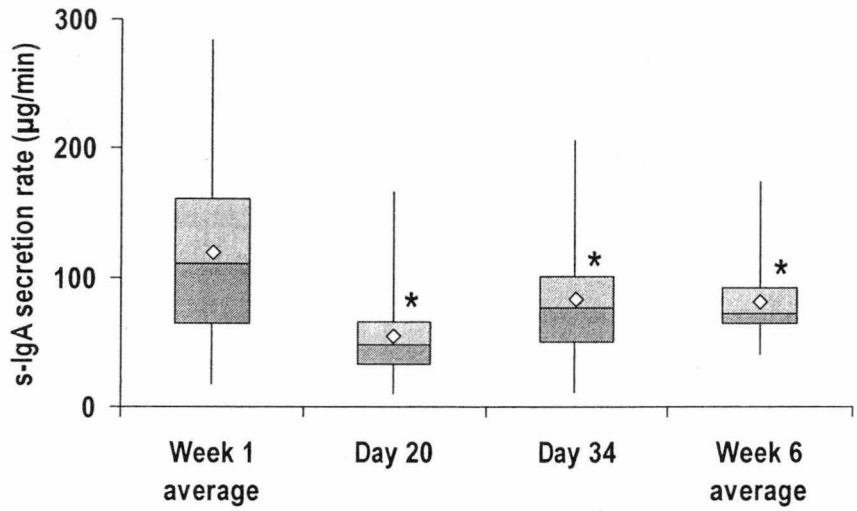
Table 25: Biological variation of late afternoon s-IgA during week 1 of the ACRT

s-IgA measure	CV_I	CV_G	II
IgA secretion rate ($\mu g \cdot min^{-1}$)	53% (± 25%)	80%	0.66
IgA:osmolality ratio ($mg \cdot mOsmol^{-1}$)	42% (± 16%)	58%	0.72

5.3.3 Changes in salivary IgA during the ACRT

There was strong evidence that the measures of s-IgA changed across the selected set of sample time points (week 1 average, day 20, day 34, and week 6 average): pre-dinner s-IgA secretion rate ($F_{27,3} = 14.356$, $P < 0.001$) and s-

IgA:osmolality ratio ($F_{25,3} = 55.373$, $P < 0.001$). The statistical analysis was performed on the mean-of-the-means for weeks 1 and 6 and on the group mean for days 20 and 34. The nature of change was similar for both s-IgA measures, a decrease from week 1 to day 20 that was sustained until week 6 (Table 26, Figure 13). Both s-IgA measures were lowest on day 20. There were no differences between day 34 and week 6. The raw data for all subjects has been included in table format in Appendix I.



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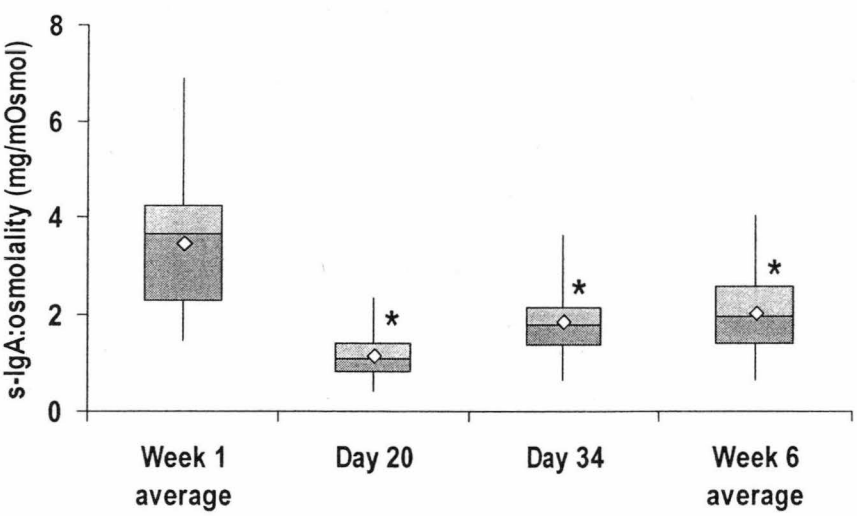

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Figure 13: *Changes in pre-dinner s-IgA secretion rate (a) and s-IgA:osmolality ratio (b) during the ACRT. Both had decreased by day 20. On day 34 and during week 6 all measures of s-IgA were higher than on day 20, but still lower than during week 1. * Indicates a significant difference with week 1 ($P < 0.05$). The statistical analysis was performed on the mean-of-the-means for weeks 1 and 6 and on the group mean for days 20 and 34.*

Table 26: Changes in pre-dinner s-IgA measures over the course of the ACRT

s-IgA measure	Average week 1	Day 20	Day 34	Average week 6
s-IgA secretion rate	120 ± 69	55 ± 35*	83 ± 49#	81 ± 32#
(μg·min^{-1})	(94 – 145)	(42 – 68)	(64 –101)	(70 – 93)
s-IgA:osmolality ratio	3.4 ± 1.3	1.1 ± 0.5*	1.9 ± 0.7*	2.0 ± 0.8*
(mg·mOsmol^{-1})	(2.9 – 3.9)	(0.9 – 1.4)	(1.6 – 2.1)	(1.7 – 2.3)

Data are represented as mean ± *SD* (95% confidence interval); * $P < 0.001$; ** $P < 0.005$; # $P < 0.05$, significant change from week 1 (N = 28-30).

The patterns displayed in Figure 13 are based on mean s-IgA levels for all subjects. Given that the practical interest lies in assessing changes in an individual's s-IgA levels as well as from a group of subjects, the temporal correlations for the various pre-dinner s-IgA measures were assessed. High temporal correlations would mean there was a consistent change over time among individuals. The fact that correlations for an individual across time were not high for any of the variables (Table 27) indicates that the strong pattern observed using mean responses for all subjects was not consistent across individuals.

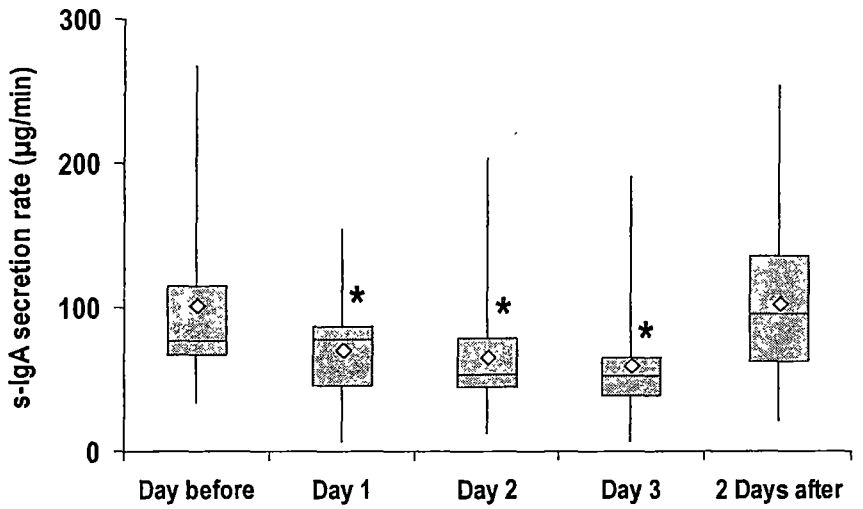
Table 27: Correlations across time for pre-dinner s-IgA variables (N = 28-30)

s-IgA secretion rate	Week 1 average	Day 20	Day 34	Week 6 average
Week 1 average	1.0	0.5	0.7	0.2
Day 20		1.0	0.4	0.3
Day 34			1.0	0.5
Week 6 average				1.0
s-IgA:osmolality ratio	**Week 1 average**	**Day 20**	**Day 34**	**Week 6 average**
Week 1 average	1.0	0.5	0.6	0.5
Day 20		1.0	0.4	0.2
Day 34			1.0	0.5
Week 6 average				1.0

5.3.4 Effect of Exercise Dusty Warrior on salivary IgA

During the period of EX DW, there was no change in pre-breakfast s-IgA secretion rate ($F_{4,68}$ = 1.296,P = 0.280) or s-IgA:osmolality ratio ($F_{4,60}$ = 1.716 ,P = 0.158). There was, however, a significant decline in pre-dinner s-IgA secretion rate ($F_{4,104}$ = 5.933, P < 0.001) and s-IgA:osmolality ratio ($F_{3\,5,\,88\,3}$ = 6.458, P < 0.001) (Table 28, Figure 14).

Compared to baseline (day before the start of EX DW), s-IgA secretion rate was lower at the end of each day. S-IgA:osmolality ratio was lower at the end of days two and three. Both had recovered two days after EX DW. The individual data for all subjects has been included in table format in Appendix J



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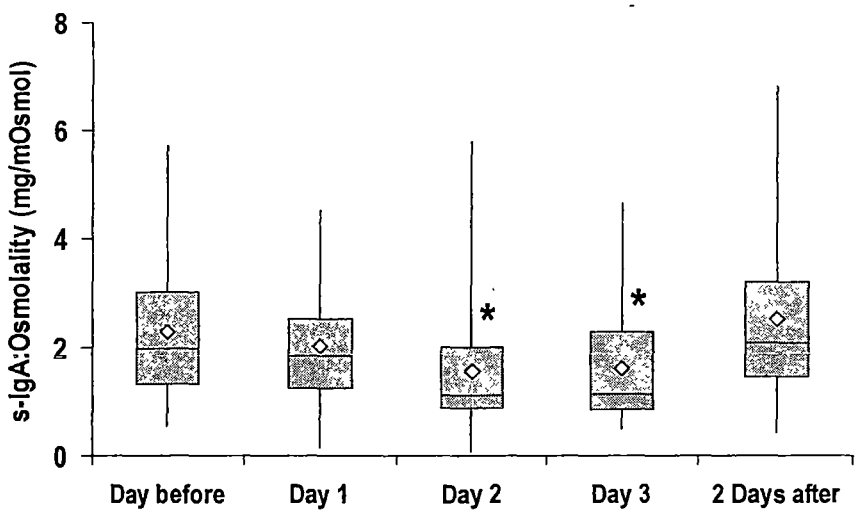

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Figure 14: *Changes in pre-dinner s-IgA secretion rate (a) and s-IgA:osmolality ratio (b) during Exercise Dusty Warrior. Both decreased, and then returned back to pre-exercise values two days after its completion. * Indicates a significant difference with the evening before the start of EX DW ($P < 0.05$). The presentation of data is similar to Figure 5.*

Table 28: Effect of Exercise Dusty Warrior on early morning and evening s-IgA measures

s-IgA measure	Baseline: day before EX DW	EX DW day 1	EX DW day 2	EX DW day 3	Recovery: 2 days after EX DW
Early morning (pre-breakfast)					
s-IgA secretion rate ($\mu g \cdot min^{-1}$)	95 ± 56 (71 - 120)	131 ± 79 101 - 162)	88 ± 45 (67 - 105)	72 (54 - 96)	92 ± 47 (72 - 112)
s-IgA:osmolality ratio ($mg \cdot mOsmol^{-1}$)	2.5 (2.1 - 3.1)	3.9 ± 1.9 (3.1 - 4.6)	2.6 ± 1.2 (2.1 - 3.0)	2.5 ± 1.5 (1.9 - 3.1)	3.2 ± 1.6 (2.6 - 3.2)
Evening (pre-dinner)					
s-IgA secretion rate ($\mu g \cdot min^{-1}$)	87 (72 - 107)	70 ± 34* (57 - 83)	55* (44 - 69)	51* (41 - 64)	103 ± 54 (82 - 123)
s-IgA:osmolality ratio ($mg \cdot mOsmol^{-1}$)	1.9 (1.6 - 2.4)	1.7 (1.3 - 2.2)	1.2* (0.9 - 1.6)	1.3* (1.0 - 1.7)	2.1 (1.7 - 2.7)

Normally distributed data are represented as mean ± SD (95% confidence interval). Not normally distributed data are reported as geometric mean (95% confidence interval). * Significant difference with the day before Exercise Dusty Warrior ($P < 0.05$) ($N = 30$).

5.3.5 Effect of the Challenge on salivary IgA

Participation in The Challenge caused a reduction in the s-IgA:osmolality ratio ($P < 0.001$). However, there was no change in the s-IgA secretion rate ($P = 0.487$) (Table 29).

Table 29: Effect of the Challenge on s-IgA measures ($N = 30$).

s-IgA measure	Pre-Challenge	Post-Challenge
s-IgA secretion rate ($\mu g \cdot min^{-1}$)	84 (62 – 114)	77 (59 – 101)
s-IgA:osmolality ratio ($mg \cdot mOsmol^{-1}$)	2.9 (2.3 – 3.7)	1.6* (1.3 – 2.1)

Data are reported as geometric mean (95% confidence interval). * Significant difference with pre-Challenge ($P < 0.05$).

5.3.6 URTI

Of the 30 subjects who donated saliva samples, 18 reported self-assessed symptoms of URTI or influenza (Table 30) between days 1 and 38. The recording of health problems was considered unreliable after day 38, as subjects became reluctant to fill in forms conscientiously (see Appendix G). The individual data for all subjects have been included in table format in Appendix K. There were no significant correlations (as assessed by Pearson's correlation coefficient) between the total number of URTI and

influenza symptoms between days 1 and 33 (initial training period) or between days 34 and 38 (final training period) and any of the s-IgA measures on day 1, day 34, week 1 or week 6 except for a weak correlation between these symptoms in the day 1 to 33 period and the s-IgA secretion rate on day 34 ($P = 0.041$, r = 0.381).

Table 30: Number of days with self-recorded URTI and influenza symptoms per recruit during initial and final training periods for all subjects (N = 30)

Number of days	Days 1 - 33 Mean, median (range)	Days 34 -38 Mean, median (range)
Number of days with URTI symptoms	5.6, 3.5 (0 – 31)	0.3, 0 (0 – 5)
Number of days with flu symptoms	0.7, 0 (0 – 11)	None recorded

5.3.7 Indicators of overtraining

5.3.7.1 Fatigue

General fatigue ($P < 0.001$), physical fatigue ($P = 0.001$), emotional fatigue ($P = 0.002$) and mental fatigue ($P = 0.013$) had increased significantly by day 6 of the ACRT course but there was no change in vigour (Table 31). At the end of the course, general fatigue and physical fatigue were still elevated, whereas emotional, mental and total fatigue were not different from day 1. The individual fatigue scores for all subjects have been included in table format in Appendix K.

Table 31: Fatigue during the ACRT as measured by Multidimensional Fatigue Symptom Inventory-Short Form

Psychological factor	Day 1	Day 6	Day 13	Day 20	Day 27	Day 34
Vigour	16.7 ± 4.7	16.1 ± 4.4	16.8 ± 5.1	17.0 ± 4.5	15.7 ± 5.0	15.5 ± 5.0
General fatigue	10.3 ± 4.7	14.2 ± 5.0*	11.8 ± 5.1	11.6 ± 5.2	11.4 ± 4.5	13.1 ± 6.5*
Physical fatigue	8.4 ± 2.6	12.0 ± 5.1*	9.9 ± 4.0	11.2 ± 4.5*	10.7 ± 4.7*	11.8 ± 5.3*
Emotional fatigue	10.4 ± 4.1	11.4 ± 5.2	9.4 ± 4.5	8.9 ± 3.4*	9.3 ± 3.7*	9.5 ± 4.6
Mental fatigue	10.5 ± 4.4	13.4 ± 4.3*	11.7 ± 4.2	11.0 ± 3. 8	10.9 ± 4.2	10.7 ± 4.7
Total fatigue	11.3 ± 5.0	13.4 ± 5.1	11.9 ± 5.2	12.0 ± 5.1	11.6 ± 4.9	12.1 ± 5.6

Data are mean ± *SD*; * Significant difference with day 1 ($P < 0.05$).

5.3.7.2 Mood

There were significant changes in POMS scores for tension-anxiety ($P < 0.001$), depression-dejection ($P = 0.026$), anger-hostility ($P = 0.004$), fatigue-inertia ($P < 0.001$), confusion-bewilderment ($P < 0.001$) and total mood disturbance ($P = 0.022$) during the ACRT (Table 32). All were highest early during the course (day 6) with the exception of tension-anxiety. The individual POMS scores for all subjects have been included in table format in Appendix K.

Table 32: Changes in negative mood states during the ACRT as measured by POMS

Psychological factor	Day 1	Day 6	Day 13	Day 20	Day 27	Day 34
Tension-anxiety	12.3 ± 5.4	11.9 ± 6.4	7.3 ± 4.5*	7.0 ± 4.5*	6.4 ± 4.2*	5.9 ± 3.7*
Depression-dejection	7.1 ± 10.0	10.0 ±10.0*	4.8 ± 7.6*	5.9 ± 7.7	6.5 ± 9.0	6.9 ± 9.8
Anger-hostility	4.8 ± 6.3	8.9 ± 7.5*	5.3 ± 6.7	6.0 ± 6.8	5.8 ± 7.0	6.2 ± 6.4
Fatigue-inertia	6.8 ± 6.1	9.5 ± 5.2*	6.4 ± 5.4	6.8 ± 6.2	5.3 ± 5.2	6.9 ± 7.2
Confusion-bewilderment	7.3 ± 4.5	8.9 ± 4.6*	6.8 ± 3.8	6.1 ± 3.7*	5.2 ± 3.9*	5.6 ± 3.5*
Total mood disorder	22.3 ± 33.6	32.9 ± 28.7	12.9 ± 26.1	17.4 ± 28.1	13.3 ± 27.9	16.0 ± 29.5

Data are mean ± *SD*; * Significant change from day 1 ($P < 0.05$).

5.3.7.3 Hormonal changes and markers of inflammation

There was a gradual decrease in both free testosterone and cortisol during the ACRT that became significant at the last sample point ($P < 0.001$) (Table 33). At the end of the ACRT, 24 of the recruits (56%), including 1 female experienced a decline in FTCR greater than 30%, which has been suggested as being symptomatic of OTS. There was a significant increase in the serum concentration of C-reactive protein (CRP) ($P = 0.001$) and tissue necrosis factor α (TNF_α) ($P = 0.002$) during the ACRT (Table 33) which have also been implicated with OTS. The individual data for all subjects have been included in table format in Appendix K.

5.3.7.4 Humoral immune function

Total white blood cell count, neutrophil, lymphocyte and monocyte count declined from day 1 to day 33 of the ACRT, but there was no change in these measures between days 33 & 43 (Table 33). There was also a decrease in the neutrophil to lymphocyte ratio between days 1 & 33 ($P = 0.003$). The individual data for all subjects have been included in table format in Appendix K.

Table 33: Changes in hormonal and humoral indicators of overtraining and inflammation during the ACRT

Variable	Day 1	Day 33	Day 43
Free testosterone (males)	67 ± 23	43 ± 15*	37 ± 11**
(pmol·L^{-1}) (N = 37)	(26 – 118)	(10 – 88)	(10 – 57)
Cortisol (nmol·L^{-1})	469 ± 142	438 ± 63	413 ± 72*
(N = 44)	(196 – 750)	(293 – 602)	(298 – 578)
FTCR (males)	0.15 ± 0.10	0.10 ± 0.04*	0.09 ± 0.03*
(N = 37)	(0.04 - 0.40)	(0.02 - 0.21)	(0.02 - 0.14)
C-reactive protein (mg·L^{-1})	1.2 ± 1.7	1.6 ± 2.9	2.1 ± 3.7*
(N = 41)	(0.2 - 9.4)	(0.2 - 17.1)	(0.3 - 23.5)
Tissue necrosis factor α (µg·L^{-1})	2.8 ± 1.3	3.2 ± 1.9	4.8 ± 2.7*
(N = 40)	(0.9 - 5.7)	(0.9 - 10.6)	(1.6 - 14.7)
Total leukocyte count (x 10^9)	8.1 ± 1.9	6.1 ± 1.4*	6.3 ± 1.4*
(N = 40)	(4.7 - 11.9)	(2.6 - 9.2)	(3.5 - 9.8)
Neutrophil count (x 10^9)	5.5 ± 2.2	3.3 ± 0.9*	3.6 ± 1.0*
(N = 37)	(2.1 - 12.9)	(1.5 - 5.8)	(1.9 - 6.1)
Lymphocyte count (x 10^9)	1.8 ± 0.5	1.6 ± 0.4*	1.7 ± 0.4
(N = 43)	(0.9 - 3.1)	(1.0 - 2.8)	(1.2 - 2.8)
Monocyte count (x 10^9)	0.8 ± 0.4	0.6 ± 0.1*	0.6 ± 0.2*
(N = 38)	(0.4 - 2.3)	(0.3 - 1.0)	(0.3 - 1.2)
Neutrophil to lymphocyte ratio	3.3 ± 1.7	2.3 ± 1.5*	2.1 ± 0.8*
(N = 43)	(1.2 - 8.6)	(0.8 - 9.5)	(0.3 - 4.2)
Haemoglobin males (g·L^{-1})	154 ± 9	141 ± 8*	135 ± 9**
(N = 38)	(134 – 174)	(120 – 158)	(116 – 161)
Haemoglobin females (g·L^{-1})	137 ± 2	126 ± 3*	125 ± 4*
(N = 5)	(134 – 138)	(120 – 128)	(120 – 130)
Ferritin-males (µg·L^{-1})	120 ± 67	72 ± 31*	85 ± 37*
(N = 38)	(38 – 368)	(31 – 171)	(24 – 171)
Ferritin-females (µg·L^{-1})	67 ± 30	39 ± 10*	48 ± 5*
(N = 5)	(34 – 105)	(27 – 53)	(43 – 55)

All data are mean ± *SD* (range); * significant change from day 1; ** significant change from day 33 ($P < 0.05$). Unless otherwise specified data are for males and females combined.

5.3.7.5 Haematology and iron status measurements

There was a consistent decline in haemoglobin and ferritin during the ACRT ($p < 0.001$) (Table 33). Ferritin (Frt) levels were significantly higher in males than in females at all sample points ($P < 0.001$), however, there was no difference in the pattern of change between males and females. These findings must be treated with caution as there

were only 5 females in this analysis. The individual data for all subjects have been included in table format in Appendix K.

5.3.8 Salivary IgA and indicators of overtraining

To assess if s-IgA measures were correlated with the indicators of overtraining on corresponding days, linear regression analyses for the variables listed in Table 34 were used. The left hand column shows the psychological, haematological and biochemical response variables (e.g. general fatigue and cortisol) which were correlated with the s-IgA secretion rate and s-IgA:osmolality ratio as predictor variables (in the right hand column). Because this resulted in a very large number of comparisons, only those which resulted in a significant correlation were included in Table 35. The high levels of physical fatigue and mental fatigue were not correlated with s-IgA throughout the ACRT. The high levels of depression, anger and confusion at the end of the 1st week were not correlated with any of the s-IgA measures. Correlations with other indicators of overtraining including hormonal changes, an increase in inflammation, and a decreased iron status, were equally absent or inconsistent. Although more than half the recruits experienced a clinically significant decline in FTCR of greater than 30%, this was not correlated to s-IgA at all. Although TNFα was positively correlated s-IgA:osmolality ratio on day 33, this occurred before the increase in TNFα. There were only a few weak correlations between the haematological parameters and s-IgA.

To evaluate if changes in s-IgA predicted changes in indicators of overtraining, the difference between the initial test result and each of the results at subsequent testing times were treated as a response variable and the two s-IgA variables as predictor variables in a multiple linear regression equation. Using stepwise fitting, only the variables for which a significant relationships were established are listed in Table 36 together with the percentage of variation explained. The same regression analysis method was used to determine if the baseline values of s-IgA measures (average of week 1) were predictive of changes in other variables. Only the analyses that produced significant associations are listed in Table 37. Only a small percentage of the changes in a few indicators of overtraining during the ACRT were explained by the baseline (week 1) s-IgA measures or by changes in s-IgA measures.

Table 34: List of psychological, haematological and biochemical variables that were used as response variables and s-IgA predictor variables (or the base variables from which response and predictor variables were calculated)

Response variable			Predictor variables: s-IgA secretion rate; s-IgA:osmolality ratio
Physiological	**Weight**	Day 1, day 43	Week 1 average, week 6 average
Fatigue	**General**	Days 1, 6, 13, 20, 27, 34	Week 1 average, days 6, 13, 20, 27, 34, week 6 average
	Physical		
	Emotional		
	Mental		
	Vigour		
Mood (POMS)	**Tension**		
	Depression		
	Anger		
	Fatigue		
	Confusion		
	Total mood		
Markers of inflammation	**C-reactive protein**	Days 1, 33, 43	Week 1 average, day 34, week 6 average
	TNF_{α}		
Haematology	**haemoglobin**		
	Ferritin		
Hormones	**Free testosterone**		
	Cortisol		
	FTCR		
Immunological	**Leukocyte count**		
	Neutrophil count		
	Monocyte count		
	Neutrophil:lymphocyte ratio		

Table 35: Significant correlations between s-IgA and indicators of overtraining on corresponding days during the ARTC

Day	Predictor variables	Response variables	*r*	*P*
1	s-IgA secretion rate	Cortisol	- 0.455	0.017
33	s-IgA:osmolality ratio	TNF_{α}	0.505	0.009
1	s-IgA secretion rate	Total leukocytes	0.461	0.020
1	s-IgA:osmolality ratio	Total leukocytes	0.409	0.047
1	s-IgA:osmolality ratio	Neutrophils	0.475	0.017
1	s-IgA:osmolality ratio	Lymphocytes	0.462	0.020
33	s-IgA:osmolality ratio	Ferritin	-0.421	0.029

Table 36: Changes in s-IgA measures that predicted changes in indicators of overtraining during the ACRT

Predictor variable	Response variable	% variation explained (R^2)*
Change in s-IgA secretion rate - week 1 to day 34	Change in physical fatigue - day 1 to day 34	12
Change in s-IgA:osmolality ratio - week 1 to day 34	Change in total mood - day 1 to day 34	19
Change in s-IgA secretion rate – week 1 to day 34	Change in neutrophil count - day 1 to day 33	22
Change in s-IgA secretion rate – week 1 to day 34	Change in neutrophil:lymphocyte ratio - day 1 to day 33	12
Change in s-IgA secretion rate – week 1 to week 6	Change in ferritin - day 1 to day 43#	26

* Percentage of variation observed in the response variable associated with the variation in the s-IgA measure.
# Unfortunately (but beyond our control) there was a one day lag between the blood sample (day 33) and the saliva sample (day 34).

Table 37: Baseline s-IgA measures that predicted changes in indicators of overtraining during the ACRT

Predictor variable (average of week 1)	Response variable	% variation explained (R^2)*
s-IgA:osmolality ratio	Change in total mood - day 1 to day 13	27
s-IgA secretion rate	Change in white blood cell count - day 1 to day 33	28
s- IgA secretion rate	Change in neutrophil count - day 1 to day 43	28
s-IgA secretion	Change in cortisol - day 1 to day 33	14
s-IgA secretion	Change in FTCR - day 1 to day 33	16
s-IgA secretion	Change in FTCR - day 1 to day 43	15

* Percentage of variation observed in the response variable associated with the baseline value of the s-IgA measure

5.4 Discussion

The subjects in this study engaged in daily physical activity over a six week period, some of which was prolonged and/or intense. Indeed, the mean daily energy expenditure for the ACRT course was 16.5 MJ. This is similar to the energy expenditure determined by doubly-labelled water method during 12 weeks of strenuous US marine corps training (17 ± 4 MJ·day^{-1} for males and 10 ± 1.3 MJ·day^{-1} for females) (Bathalon et al., 2002). The workload was highest at the end of the course when the Challenge and EX DW were conducted. On the most arduous day of EX DW the energy expenditure was approximately 28 MJ for male recruits. On any day recruits were probably engaging in a greater level of physical activity than would have been normal for them prior to their participation in the ACRT. The recruit's average energy expenditure of 16.5MJ would be considered very high for their relatively sedentary civilian peers. It was, therefore, postulated that the subjects would present with some symptoms of overtraining. It was also postulated that the ACRT would result in decreased levels of s-IgA. Investigating how these changes in s-IgA would relate to the indicators of overtraining was the main purpose of this study.

Although the results suggest that in general recruits responded favourably to physical fitness training, there was some evidence for a decline in aerobic endurance by the last week of the course. This could have been due to the higher levels of physical fatigue and might be suggestive of some overtraining. The officers and instructor of ARTC expressed the concern that recruits were having difficulty maintaining concentration throughout the day and were taking a long time to recover after physically

demanding activities (personal communication). The tests of physical performance used in this study gave only a rough estimate of the changes in fitness of the recruits and are not necessarily reliable. Testing was conducted by the ARTC physical fitness instructors rather than by the investigators and the methods for recording results and time-of-day were not standardised. It was impossible to ascertain if recruits performed to the maximum of their ability. For instance if the subjects were losing their motivation by the end of the trial, the results of a 2.4 km run would not have given a good indication of maximal aerobic power.

The small loss of body mass experienced by most recruits was not clinically significant. At the end of the ACRT, no recruits had an unhealthy BMI, and without data on body composition, the extent of fat or muscle loss could not be estimated.

All fatigue variables showed a peak in the second week of the ACRT and general fatigue and physical fatigue were elevated at the end of the sixth week (Booth et al., 2006; Skiller et al., 2005b). This was expected given the increasing demands of the course and the effect of an accumulation of reduced sleep. During the most strenuous days at the end of the ACRT (especially week 6), there were significantly less hours available for sleep.

After a fairly easy first week, the ACRT course becomes more challenging in the second week. This was reflected by a peak in the negative mood scores (depression, anger, fatigue, confusion and total mood disorder) during that week, indicating an increase in stress (Booth et al., 2006; Skiller et al., 2005b). This might be desirable to achieve the required training effect as the ACRT is deliberately challenging in order to indoctrinate recruits. The decrease in negative mood scores in the following weeks indicated that the training tempo/stress was moderated in subsequent weeks. Mood disturbances have been shown to increase with training load in an apparently dose-related manner (Morgan et al., 1988) and an athlete's mood state is at present considered to be a promising indicator of overtraining (McKenzie, 1999).

The findings of the previous chapters in the present work indicate that physical activity of different duration and intensity, such as ultra-endurance runs (Chapter 3) and load carriage (Chapter 4), have a transient effect on mucosal immunity. Not surprisingly, the ACRT course as a whole and several of its individual components resulted in significant (albeit transient) changes in s-IgA. By day 20, all s-IgA measures were below the week 1 average, and although they recovered partly, they remained low until the end of the course. This finding confirms that of previous studies. In Canada, an 18.5 week infantry training program resulted in lower s-IgA concentration at the end of

the course (Brenner et al., 2000) and s-IgA concentration and s-IgA:protein ratio declined after a 5-day military course following 3 weeks of combat training in France (Gomez-Merino et al., 2003). In the present study, the patterns for individuals showed considerable variation and the large between- and within-subject biological variation observed in the first week were similar to those reported in the literature and similar to the findings in Chapter 2 (Table 2 and Table 3).

After the fitness testing on day 32, the s-IgA secretion rate was significantly lower than pre-test. The strenuous Exercise Dusty Warrior resulted in significant declines in all late afternoon s-IgA measures, although this was not the case for the s-IgA measures taken in the early morning. Without exception, pre-breakfast s-IgA measures were higher than the previous evening. As there was no proof (see Chapter 2) that early morning s-IgA is necessarily higher, this suggests that there was an overnight recovery. A day later, the Challenge caused a decrease in s-IgA:osmolality but not in the secretion rate. Two days after completion of EX DW, all values had returned to the levels measured a day before its start.

The low number of subjects makes it difficult to make very useful deductions about the correlation between changes in s-IgA and the occurrence of URTI. Data on URTI were obtained from daily self-report questionnaires and were not assessed by a physician. At no stage of the study was the investigator present when these forms were filled out. Therefore it is difficult to discuss the nature of these symptoms and whether the URTI were truly infections or more likely a response to inflammation. Although a considerable number of recruits reported symptoms of URTI and influenza, there was no evidence that those with a depressed mucosal immune function at the start of the ACRT had a greater risk of URTI during the ACRT. Baseline (week 1) s-IgA measures were not correlated to URTI symptoms during the ACRT. The positive correlation between symptoms of URTI and influenza in the period from day 1 to day 34 and the s-IgA secretion rate on day 34 ($P = 0.041$, $r = 0.381$; see section 5.3.6) suggest there was an immune response in the form of an increase in antibody secretion in recruits with URTI symptoms. In similar studies, an increase in URTI in French Army cadets during a 4 week commando course was not related to s-IgA (Tiollier et al., 2005) and an 18.5-week infantry training program had no adverse effect on the health status of Canadian recruits despite a decrease in s-IgA concentration by the end of the course (Brenner et al., 2000).

Increased confusion is a common symptom reported by overtrained individuals (Mackinnon, 2000). The mean level of confusion was higher than that experienced by personnel completing either the Royal Australian Air Force survival course (Carins and

Booth, 2002) or a combat training exercise in far North Queensland (Booth et al., 2003). The other negative mood scores in this study were similar to these other military activities despite a lower score for fatigue (Booth et al., 2006; Skiller et al., 2005b).

Over the course of the ACRT 56% of the recruits experienced a decrease in FTCR of more than 30% which is higher than the 24% incidence reported for a similar 8-week recruit-training program conducted in Spain (Chicharro et al., 1998a). As prolonged intense training of initially unfit individuals is most likely to cause a drop in circulating serum hormone levels and to affect FTCR (Adlercreutz et al., 1986), the data of this study suggest that some of the recruits did not have sufficient initial fitness to cope with the demands of the ACRT and possibly were overtrained by the end of the six week period.

The intense physical activity during the final and most strenuous week of the ACRT course clearly elicited an inflammatory response as evidenced by the marked increase in the serum concentration of markers of inflammation (CRP, TNFα) (Booth et al., 2006; Skiller et al., 2005b). This finding is consistent with the so-called cytokine hypothesis of overtraining (Smith, 2000), which argues that high tempo training without adequate rest will result in muscle and/or joint trauma and a release of injury-related cytokines. These cytokines then activate monocytes to produce large quantities of pro-inflammatory cytokines (e.g. TNF_{α}) producing systemic inflammation, an acute phase response with production of proteins such as CRP and a hypercatabolic state, all of which have a negative impact on immune function.

Markers of humoral immune function (total leukocytes, neutrophils, monocytes, and the neutrophil:lymphocyte ratio) had declined by the end of week 5 of the ACRT course (Booth et al., 2006; Skiller et al., 2005b). This is contrary to the observations of Gomez-Merino et al who found that total leukocyte and neutrophil counts had increased while total lymphocytes were unchanged after three weeks of basic military training followed by a 5-day combat course (Gomez-Merino et al., 2005). Although suppression of humoral immune function during prolonged periods of intense exercise training is a common finding - but not necessarily indicative of overtraining (Mackinnon, 2000) - it is rare for individuals to become clinically immunosupressed during regular physical training. The most likely risk is an increased incidence of URTI (see 1.5). The negative correlation between the self-recorded symptoms URTI and the neutrophil to lymphocyte ratio at the start of the ACRT suggests that recruits with decreased immune function at the start of the course were indeed more likely to experience URTI.

Both mean haemoglobin and serum ferritin concentrations declined across the training period, with some evidence for recruits becoming iron deficient (Booth et al., 2006; Skiller et al., 2005b). This is in accord with previous studies which have shown that the iron-status of soldiers declines over the course of routine military field exercises (Booth, 2003; Booth et al., 2003). Although mild anaemia, low serum ferritin levels and iron-deficiency have been suggested as physiological indicators of overtraining (Gastmann et al., 1998; Mackinnon, 2000), the evidence that a reduction in iron stores, associated with increased exercise, adversely affects physical performance is equivocal, except where iron deficiency anaemia is present.

The results of this study do not support the suggestion that s-IgA would be useful as a single biological marker to detect overtraining (McKenzie, 1999). There is no doubt that, as a result of the ACRT, recruits experienced several symptoms which have been associated with overtraining (Booth et al., 2006; Skiller et al., 2005b) and that all s-IgA measures were depressed from day 20 onwards. However, despite the fact that changes in s-IgA measures seemed to reflect changes in most of the indicators of overtraining, only limited and inconsistent associations were found between them.

A few examples illustrate this. The high levels of physical fatigue and mental fatigue were not correlated with s-IgA throughout the ACRT. The high levels of depression, anger and confusion at the end of the 1st week were not correlated with any of the s-IgA measures. Friendliness was correlated positively with s-IgA secretion rate and s-IgA:osmolality ratio on day 13 only. Correlations with other indicators of overtraining including hormonal changes, an increase in inflammation, and a decreased iron status, were equally absent or inconsistent. Although more than half the recruits experienced a clinically significant decline in FTCR of greater than 30%, a criterion used previously to diagnose OTS, this was not correlated to s-IgA at all. Although TNF_{α} was positively correlated to s-IgA concentration and s-IgA:osmolality ratio on day 33, this occurred before the increase in TNF_{α}. Only a small percentage of the changes in a few indicators of overtraining during the ACRT were explained by the baseline (week 1) s-IgA measures or by changes in s-IgA measures (Table 36 and Table 37). It is not inconceivable that many, if not all, of the relations listed in these 2 tables could have arisen by chance and as a result of sampling variation rather than being indicators of real associations. None of the associations were strong enough to suggest that any of the s-IgA values (baseline or otherwise) or changes over time in s-IgA would be useful as a predictor of indicators of OTS or their changes across time. Such relationships as existed showed a level of association that is far too low to be of practical use. For any s-

IgA measure to be reliable as a predictor of any response variable an R^2 value in excess of 95% would be required. Therefore, it does not seem appropriate to use s-IgA as an alternative to any of these other indicators.

Nevertheless, the fact that any associations were found at all, given the restrictions of the experimental design, is encouraging. To obtain a better understanding of the relationship between s-IgA and indicators of OTS further research seems warranted. As the large within-subject and between-subject biological variation of s-IgA reduces the likelihood that significant changes in an individual become apparent, a much larger group of subjects and a much larger number of saliva samples per individual would be required.

A design flaw of this study is that contrary to the recommendation made in Chapter 2 (section 2.4.6), some early morning samples were collected for this study. As the sample collection times had to fit in with the very busy schedule of the subjects (16 hour a day), these were to a large extent beyond our control. At the time the study was designed we were also unaware of the conclusions relating to biological variation as presented in Chapter 2.

It was concluded "*that the combined demands of ACRT, which included physical and psychological stresses, resulted in a significant prevalence of overtraining symptoms. However, recruits were not pushed so hard that physical performance deteriorated greatly*" (Booth et al., 2006) There was however no evidence that s-IgA is a useful biomarker to monitor recruits for the risk of infection despite the fact that the ACRT resulted in a high incidence of mucosal immunosuppression and self-reported symptoms of URTI. Although the ACRT also resulted in a significant prevalence of OTS symptoms there was no evidence that s-IgA is a useful stand-alone biomarker to monitor recruits for early states of OTS as a result of military training. Several recommendations to reduce the risk of URTI and OTS in recruits can be found in the general discussion.

GENERAL DISCUSSION AND CONCLUSION

The purpose of this project was to evaluate the usefulness of s-IgA as a biomarker of stress and immunosuppression for use in military training. A simple diagnostic test to assess the effect of physical and psychological stress on mucosal immune function and subsequent vulnerability to illness or early states of OTS would be very useful, not only to the military, but also to the exercising population at large.

As reviewed in the Introduction (Chapter 1), work by previous authors on s-IgA raised some concerns regarding its reliability and its validity as an indicator of mucosal immune function and subsequent risk or incidence of infection. Weaknesses identified were the possible effects of diurnal variation (section 1.3.7), the large biological variation (sections 1.3.8 and 1.3.9), the standardisation of the s-IgA assay (section 1.3.3), and the collection, handling and storage methods of saliva samples (sections 1.3.4 and 1.3.5). These issues were addressed by several preliminary experiments.

At present, there are no quick, simple and inexpensive field- or laboratory-testing methods for the detection of s-IgA. Using s-IgA as a biomarker for regular routine testing would only be feasible if a robust and cheap method of analysis was available. The development of such a method should be possible as antisera to human IgA are readily obtainable and s-IgA was shown to be stable for a limited time under field conditions (section 1.3.5). This study found that the measurement of s-IgA by particle enhanced nephelometric immunoassay is a good alternative to other methods as it is automated, rapid, accurate and precise. Care needs to be taken when collecting, storing and analysing saliva samples.

This study does not offer an explanation for the large biological variation that seems inherent to this marker. Because it is unlikely that any study will be able to control each of the many possible contributing factors, one will always have to take into account that the changes in s-IgA observed as a result of, for instance physical exercise, may in fact be largely due to normal biological variation. The large degree of uncertainty of any s-IgA measurement is therefore its main drawback. Even though pre-analytical and analytical variation can be minimised, the large biological variation has serious implications when changes in s-IgA are interpreted as large changes are required for serial results to be statistically and biologically different.

Although no proof of a diurnal variation in s-IgA was found, it is recommended that the time of day at which saliva samples are taken should be accurately recorded and that repeat samples should be collected at the same time of day if required for comparison. The degree of uncertainty can be reduced somewhat by obtaining samples

at the time of least variability, which seems to be around mid-day. As there was a significant post-prandial effect, it is recommended that saliva samples are taken either before a meal or at least 1.5-2 hours post-prandial. Samples immediately after a meal, early morning (fasting) and late evening should be avoided because of the larger variability at these times, unless required for specific reasons.

As there is a wide range in the biological variation of s-IgA between individuals, evaluating an individual's s-IgA levels against a population based reference interval is of limited use. The establishment of a personal reference interval seems more appropriate to determine "normal" values and to detect significant changes in serial measurements for individuals. The usefulness of personal reference intervals needs to be proven and would necessitate further study. Originally it was planned to undertake a project to establish personal reference intervals for a group of subjects. However it was estimated that a very large number of samples would be needed from each subject to obtain a valid reference range. This was beyond the scope of this project. Although the recommendation is made for future research, this would require considerable effort and expense, and therefore it is doubtful that this method will become used widely.

S-IgA levels can be expressed in different ways (section 1.3.6). The results of this study confirm that s-IgA secretion rate and s-IgA:osmolality ratio are the most valid and meaningful. S-IgA secretion rate most accurately describes the amount of s-IgA available at the mucosal surface and by expressing s-IgA as a ratio to osmolality, changes in saliva flow and evaporation are corrected for. In addition, the biological variation of the s-IgA:osmolality ratio was found to be among the lowest.

The various experiments of this study confirmed previous findings that physical stress can result in significant changes in mucosal and humoral immunity. There was a transient decrease in s-IgA (and changes in several other immune parameters) in response to an 82 km ultra-endurance run, two hours of heavy load carriage at a constant speed, and during a six week army common recruit training course. Nevertheless, more study is needed to determine the effect of volume, duration and intensity of physical activity on s-IgA, and at what combination of these factors exercise becomes a negative stressor. Although the results of the CMR study suggest that mucosal immunosuppression can occur as a result of long-duration, moderate intensity exercise, only the hottest, most humid environment resulted in a significant decline of s-IgA during the load carriage study. It seems that if exercise at an intensity that by itself does not cause a significant mucosal immunosuppression is performed in a harsh climate, the resulting hyperthermia can magnify the s-IgA response. In practical terms,

this indicates that by keeping the exercise intensity in a hot and humid environment constant, the additional physiological and psychological strain will possibly lead to a greater risk of immunosuppression. Alternatively, allowing someone to adjust the exercise intensity (by slowing down or by allowing self-pacing) or to acclimate to the local environmental conditions, the strain and consequently the magnitude of the immunosuppression could possibly be reduced.

The relationship between s-IgA and the incidence of URTI remains tenuous and it remains to be proven whether there are clinical implications associated with an exercise induced reduction in s-IgA (section1.5). There was no evidence that participation in an ultra-endurance exercise like the CMR is associated with an extra risk of infection - there were few URTI symptoms pre- and post-race and there was no change in their incidence. Likewise, there was no evidence that s-IgA is a useful biomarker to monitor recruits for the risk of infection despite the fact that the ACRT resulted in a high incidence of mucosal immunosuppression and self-reported symptoms of URTI.

To investigate the potential of s-IgA as a predictor of OTS, the relationship between s-IgA and other possible indicators of OTS (e.g. fatigue; mood states; neutrophils to lymphocyte ratio; C-reactive protein; TNFα; haemoglobin; ferritin; FTCR) was examined (section 1.8). Although the ACRT resulted in a high incidence of mucosal immunosuppression and a significant prevalence of OTS symptoms (such as fatigue, immune suppression, reduced iron status, and hormonal changes), there was no evidence that s-IgA is a useful stand-alone biomarker to monitor recruits for early states of OTS as a result of military training. Further evidence is needed to prove that declining s-IgA is a sign of OTS.

In summary, although the results of these studies indicate that mucosal immunity is affected by a range of military activities, this work does not lend strong support to the notion that s-IgA could be a practical tool to monitor mucosal immune function or physiological strain in the military or that it would be a good predictor of ill health, excessive training or OTS.

Several design limitations of the projects in this study were a result of working with the military. The number of available subjects was limited and access to subjects and information was sometimes restricted because of operational considerations. More research on a larger scale is needed to determine if immune changes caused by military activities are clinically significant. Therefore, the possible usefulness of monitoring s-IgA during military activities such as load carriage or training courses should not be rejected without further investigation. A future study could for instance assess if a

modification of the ACRT course would result in maintenance or improvement of mucosal immune function in soldiers. Another study could investigate if a reduction of the load or pace of load carriage would have a similar effect. However, even if this were the case, it remains to be seen if the benefit of these changes would outweigh the disadvantages. It would make much more sense to introduce some basic strategies to maintain immunocompetence in soldiers. In order to reduce the risk of URTI and OTS in athletes, several recommendations have been made, most of which would equally apply to soldiers (Gleeson and Bishop, 2000; Nieman, 1994; Nieman, 1997; Pyne, 1999; Pyne et al., 2000; Shephard, 1998; Walsh et al., 1999). Careful management of exercise volume and intensity, variety to overcome training monotony and strain, and provision of adequate rest and recovery periods are essential. If signs and symptoms indicate that an infection is impending, the volume and intensity of training should be reduced. The tempo of recruit training could be adjusted to minimize symptoms of overtraining. For example, the high levels of mental fatigue might be avoided by providing some personal time for recreation, relaxation, or rest. Practical measures to limit the stress of exercise in the heat include: restricting training loads of soldiers until acclimatised, scheduling training sessions for early morning or late afternoon, ensuring adequate fluid replacement, wearing appropriate clothing, and educating soldiers and officers in the signs and symptoms of heat disorders. Soldiers should be taught self-management and coping skills and ways to monitor their responses to stress. Behavioural changes include adopting a well balanced diet with adequate intake of macro- and micro-nutrients; limiting transmission of contagious illnesses by reducing exposure to common infections, airborne pathogens, and physical contact with infected individuals; avoiding excessive fatigue and obtaining adequate sleep.

APPENDIX A - A selection of studies related to s-IgA and exercise from 1982 to 2005

Reference	Subjects & fitness levels	Exercise	Saliva source	Collection method	Method of analysis	Antisera	Referenced to CRM470	IgA measurement and response
(Laing et al., 2005)	12 trained cyclists	2h stationary cycling at 62% of VO_{2max}	Unstimulated whole	Salivettes	ELISA (refers to (Blannin et al., 1998))	Rabbit anti-human IgA (Sigma I-8750)	Not specified	Increase in concentration; decrease in secretion rate
(Nieman et al., 2005)	15 females accustomed to walking	30 min walking at 60% of VO_{2max}	Unstimulated whole	Expectoration	ELISA	Not specified	Not specified	No changes in concentration or secretion rate
(Tiollier et al., 2005)	21 male cadets	French army commando training	Unstimulated whole	Expectoration	Nephelometric	Not specified	Not specified	Concentration decreased after combat training
(Li and Gleeson, 2004)	8 recreationally active males	2h stationary cycling at 60% of VO_{2max}	Unstimulated whole	Expectoration	ELISA	Rabbit anti-human IgA (Sigma I-8760)	Not specified	Increased concentration; no change in secretion rate
(Palmer et al., 2003)	28 ultra-marathon runners	80 km ultra-endurance run	Unstimulated whole	Expectoration	ELISA	Not specified but refers to methods of (Gleeson et al., 1999a; Gleeson et al., 2000b; Gleeson et al., 1999b)		Decrease in concentration, secretion rate and ratio to protein
(Nieman et al., 2003)	45 runners	160 km ultra-endurance run	Unstimulated whole	Expectoration	ELISA	Not specified but refers to methods of (Gleeson et al., 1999a; Gleeson et al., 2000b))		No change in concentration and ratio to protein. Decrease in secretion rate
(Walsh et al., 2002)	15 trained male cyclists	2h stationary cycling at 70% of VO_{2max}	Unstimulated whole	Salivettes	ELISA (refers to (Blannin et al., 1998))	Rabbit anti-human IgA (Sigma I-8750)	Not specified	Decrease in secretion rate; no change in rate to osmolality
(Nieman et al., 2002)	98 competitive runners	Marathon race	Unstimulated whole	Expectoration	ELISA	Not specified (but refers to methods of (Gleeson et al., 1999a; Gleeson et al., 2000b; Gleeson et al., 1999b))		Decrease in concentration, secretion rate and ratio to protein
(Fahlman et al., 2001)	26 active females	30sec Wingate test	Unstimulated whole	Not specified	ELISA (refers to (Mackinnon et al., 1993a))	Goat anti-human IgA (Sigma)	Not specified	Decrease in secretion rate and ratio to protein; no change in concentration and ratio to osmolality
(Gleeson et al., 2000b)	22 elite swimmers	12 week training cycle	Unstimulated whole	Not specified	ELISA	Commercial IgA-specific (Tago)	Referenced against WHO 67/95 with conversion factor to CRM470	Concentration decreased after training sessions

(Fricker et al., 1999)	Case study of a well trained male swimmer	8 month training period	Not specified	Not specified	Not specified	Not specified	Not specified	Concentration decreased over study period
(Gleeson et al., 1999a)	25 elite swimmers	7 month training season	Unstimulated whole	Not specified	Electro-immunodiffusion and RID	Commercial (The Binding Site)	Not specified	Concentration decreased after training sessions
(Walsh et al., 1999)	8 well trained game players	High intensity intervals on cycle ergometer	Unstimulated whole	Dribbling	ELISA	Rabbit anti-human IgA (Sigma I-8760)	Not specified	No change in concentration & secretion rate
(Blannin et al., 1998)	18 males of mixed fitness	Cycling at 55% and 80% of VO_{2max}	Unstimulated whole	Salivettes	ELISA	Rabbit anti-human IgA (Sigma I-8760)	Not specified	Concentration and secretion rate increased; no change in ratio to protein
(Hübner-Wozniak et al., 1998)	6 elite wrestlers	16 day training camp	Unstimulated whole	Not specified	RID (referred to (Hübner-Wozniak et al., 1997))	Commercial (Dade Behring)	Not specified	No change in concentration; decrease in ratio to protein
(Ljungberg et al., 1997)	20 well trained runners	Marathon run	Unstimulated whole; stimulated whole; stimulated parotid	Drooling or "SLURP" cups	Immuno-fluor technique (Bio-Rad Laboratories)	Not specified	Not specified	Concentration & secretion rate increased in whole saliva but not in parotid saliva
(Steerenberg et al., 1997)	19 competitive & 23 recreational triathletes	Olympic distance triathlon	Stimulated whole	Expectoration	ELISA	Rabbit anti-human IgA	Not specified	Concentration unchanged; secretion rate & protein ratio decreased
(Buckwalter et al., 1996)	30 untrained women	30 min treadmill walking/jogging at 60% of HR_{max}	Unstimulated whole	Not specified	ELISA	Not specified	Not specified	Concentration increased
(Mackinnon et al., 1993a)	8 elite male kayakers	Intense interval exercise	Unstimulated whole	Not specified	ELISA	Goat anti-human IgA (Tago)	Not specified	Secretion rate & ratio to protein decreased
(Mackinnon and Jenkins, 1993)	12 young recreational males	Supra-maximal intervals on cycle ergometer	Unstimulated whole	Not specified	ELISA	Goat anti-human IgA (Tago)	Not specified	Concentration increased; secretion rate and ratio to protein decreased
(McDowell et al., 1992a)	29 moderately active male college students	Incremental treadmill to exhaustion	Unstimulated whole	Not specified	ELISA	Rabbit anti-human IgA (Sigma)	Not specified	Concentration decreased
(Housh et al., 1991)	9 males	30 min treadmill @ (80% VO_{2max}	Unstimulated whole	Not specified	ELISA	Rabbit anti-human IgA (Sigma)	Not specified	No change in concentration

(McDowell et al., 1991)	18 male college students	Treadmill tests of various duration and intensity	Unstimulated whole	Not specified	ELISA	Rabbit anti-human IgA (Sigma)	Not specified	No change in concentration
(Tharp, 1991)	27 pre- and 23 post-pubescent basketball players	Basketball practice and games	Not specified	Not specified	ELISA	Rabbit anti-human IgA (I-6008, Sigma)	Not specified	Concentration Prepubescent: no change after practice but increase after games. Postpubescent: increase 2x
(Cameron and Priddle, 1990)	13 male distance runners	10.5km fartlek run at 70-90% HR_{max}	Unstimulated whole	Not specified	Rate nephelometry	Not specified	Not specified	Concentration decreased
(Gleeson et al., 1990)	99 healthy subjects (5-75 y.o.)	Measured daily variability	Whole	Not specified	Electro-immunodiffusion	Not specified	Not specified	Considerable daily variability
(Tharp and Barnes, 1990)	21 competitive male swimmers	Several times during fall training season	Unstimulated whole	Not specified	ELISA	Non specified Sigma reagents	Not specified	Concentration decreased after each training session
(Schouten et al., 1988)	175 young recreational adults	Incremental treadmill test	Stimulated whole	Expectorating	RID	In house sheep anti-human IgA	Not specified	Concentration decreased in females but increased in males
(Mackinnon et al., 1987)	8 competitive male cyclists	2 hr cycle ergometer @ 70-75% of VO_{2max}	Stimulated parotid	Curby cap	ELISA	In house rabbit anti-human IgA	Not specified	Concentration & protein ratio decreased
(Bratthall and Widerström, 1985)	4 females & 5 males (recreational/sedentary)	15 min run at full speed	Whole stimulated	Not specified	ELISA	Not specified	Not specified	Concentration increased or decreased; secretion rate, no change; protein ratio decreased
(Tomasi et al., 1982)	8 elite cross country skiers	Cross country race	Whole & parotid	Expectoration or Curby cup	RID	In house anti-human IgA	Not specified	Concentration & protein ratio decreased

FOLLOWING APPENDIXES ARE AVAILABLE ON THE ENCLOSED CD – see back cover.

APPENDIX B: Chapter 2 - Diurnal variation - Individual results

APPENDIX C: Chapter 3 - Cradle Mountain Run - Health questionnaire

APPENDIX D: Chapter 3 - Cradle Mountain Run - Correlations with race time and training volume

APPENDIX E: Chapter 4 - Load carriage - Individual results

APPENDIX F: Chapter 5 – ACRT - MFSI-SF questionnaire

APPENDIX G: Chapter 5 – ACRT - Sleep & health diary

APPENDIX H: Chapter 5 - ACRT - Saliva biological variation - Individual results

APPENDIX I: Chapter 5 – ACRT - Changes in pre-dinner s-IgA - Individual results

APPENDIX J: Chapter 5 - ACRT – Exercise Dusty Warrior - Individual results

APPENDIX K: Chapter 5 - ACRT – Non saliva data - Individual results

APPENDIX L: Chapter 5 - ACRT - Additional figures

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